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REMOVAL OF Ca^{40} AND Ca^{45} FROM BONE BY CITRATE AS INFLUENCED BY THE PARATHYROIDS[1]

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ABSTRACT

The effects produced by the introduction of exogenous citrate in a neutralized rinsing fluid into the peritoneal cavities of nephrectomized and parathyroidectomized-nephrectomized male rats were followed through the technique of peritoneal lavage. Both groups of animals showed progressive increases in wash calcium when as little as 15 to 25 mg. of citric acid were added per 100 ml. of rinsing fluid. Peritoneal equilibration for one and one-half hours instead of one hour increased the amounts of calcium removed in the wash. Parathyroidectomized rats given the same amounts of citrate as control rats showed lower total wash calcium values; however, the increases in both groups were relative when compared to their citrate-free rinse values.

The addition of citric acid did not appreciably alter the amount of radiocalcium removed in the wash when the radioactivity was administered within twenty-four hours of the start of the lavage. Therefore, because of the increase in the total calcium removed, the specific activity of the radiocalcium in the peritoneal wash fell markedly following the citric acid administration. These experiments are interpreted as showing that citric acid mobilizes calcium from the deep areas of bone, as does the hormone of the parathyroids, and not from those areas which are in close contact with the extracellular fluid compartments. No explanation of this phenomenon can be given as yet.

IT HAS become increasingly evident during the past few years that citric acid is involved, and may play an important role in bone metabolism. Even before Dickens (1) discovered large amounts of citrate in the bone, evidence for a complex formation had been found by Shear and Kramer (2), and Kuyper (3) showed experimentally that an actual complex of calcium phosphate and citrate could be formed such as might exist in bone.

Parallel changes in calcium and citrate of blood and urine were shown to occur when either citrate or calcium was injected into experimental animals (4, 5, and 6). Fluoroacetate, known to cause the buildup of endoge-

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nous citric acid, also caused parallel increases in serum calcium (7).

Since it has been established that one of the actions of the parathyroid hormone is that of calcium mobilization, with bone as its primary target (8), it is not surprising that Alwall (9), L'Heureux and Roth (10), and Elliott and Freeman (11) found that the injection of parathyroid extract into various experimental animals produced increases in serum citrate, as well as serum calcium.

Dixon and Perkins (12) demonstrated three enzymes of the citric acid cycle in bone, indicating the presence of a possible mechanism for the production of citric acid. Carlsson *et al.* (13) recently showed that citric acid may be produced in bone under the influence of vitamin D. Neuman *et al.* (14) explained the mechanism of parathyroid hormone on bone by proposing the production of citric acid in response to parathyroid activity. They suggested that the citrate transported complexed calcium to the serum where other tissues such as the kidney released the calcium by oxidizing the citrate off the complex.

Previous reports (15, 16) from this laboratory have shown that parallel changes in calcium and citrate levels occurred in the extracellular fluid as studied by analysis of peritoneal wash in rats. The introduction of exogenous citrate in the rinsing fluid of parathyroidectomized rats was found to increase the calcium level of the equilibrated wash; however, these increases were less than those obtained when similar levels of citrate were added to the rinsing fluid in normal and nephrectomized rats.

The present experiments employed a technique using continuous peritoneal lavage, smaller amounts of exogenous citric acid, longer periods of equilibration of the rinsing fluid, and radioactive calcium. By these studies it was hoped that further information could be gained regarding parathyroid-citrate relationships and their effects on calcium metabolism.

METHODS AND MATERIALS

Male Holtzman rats weighing from 215–265 gm. were used in these experiments; however, for any one experiment, the weight range was restricted to 25 gm. The animals were maintained on a calcium deficient diet (18) three days prior to use. Nephrectomy was performed through a ventral midline incision. Parathyroidectomy involved individual removal of the glands. These operations were done under ether anesthesia 24 hours prior to use. Immediately before rinsing, a peritoneal plug made from the specifications of Talmage *et al.* (17) was stitched into the inguinal canal. The animals were kept under light nembutal anesthesia throughout the rinsing periods. Radioactive calcium was injected intraperitoneally at times as indicated in the various experiments. The amount of Ca^{45} administered varied from 10 to 50 μc but was constant for all animals in any one experiment.

The peritoneal rinsing fluid was a calcium- and phosphate-free medium containing 0.84% NaCl and 1.5% dextrose. To this, citric acid, in amounts as specified in the different experiments, was added, and the rinse adjusted to pH of 7.4. For peritoneal lavage, 30 ml. of rinsing fluid was placed in each animal and immediately removed. A second 30 ml. was placed in the cavity and allowed to equilibrate for either 1 or $1\frac{1}{2}$ hours before removal. For the sake of clarity, the word "rinse" is used to refer to the fluid placed in

the animal; the word "wash," to refer to the fluid removed from the peritoneal cavity after equilibration.

Calcium and citric acid analyses were made on the wash after removal by methods previously described (16, 18). Determinations of Ca^{45} were made directly on dried aliquots of the wash solution using a flow counter, and corrected for self-absorption. To facilitate demonstration of changes from the control condition, the average radioactivity value of the first wash of the group of rats in each experiment which were nephrectomized only was set at 1000. The individual values obtained for all washes of all animals in that experiment were then multiplied by the factor needed to correct the control value to 1000. Specific activity, as used in this report, is defined as the amount of Ca^{45}/ml. of wash divided by the mg. of calcium present in the same volume.

RESULTS

The results are presented below, divided into two series of experiments. The first group of experiments was concerned with the quantities of stable calcium removed from the bone by the addition of small amounts of citric acid to the lavage rinse. The second series dealt with the effect of exogenous citric acid on the removal of radiocalcium from bone.

A. *Effects of citric acid on the calcium removed by continuous peritoneal lavage*

1. Experiments utilizing a lavage rinse containing 15 mg. citric acid per 100 ml. and a rinse equilibration time of one and one-half hours:

For these experiments the first wash was citrate-free, and served as a control period. Calcium and citrate levels after equilibration are tabulated in Table 1. Due to dilution and metabolic breakdown of citric acid, the amount of citrate remaining in the wash at the end of each equilibration period was within the physiological range for this acid. However, it can be seen that even at this low level of exogenous citric acid administration, the amount of calcium in the wash was significantly increased. While the amount of calcium removed in each period was lower in the parathyroidectomized than in the control rats, the calcium of both groups showed the same ratio of increase when compared to values obtained in these groups after equilibration with citrate-free rinse.

2. Experiments utilizing a lavage rinse containing 25 mg. of citric acid per 100 ml. and a rinse equilibration time of one hour:

The use of this concentration of the acid in a lavage rinse which was allowed to equilibrate for only one hour had been previously reported (16) to have no effect on the calcium concentration of the equilibrated wash. However, the earlier experiments were done with the five wash periods spaced over a period of two days, in contrast to these studies utilizing a continuous peritoneal lavage covering a period of eight hours. By these improved methods, significant increases in calcium in both the control and parathyroidectomized groups could be detected by the second or third equilibration period. These data are tabulated in Table 1.

3. Experiments utilizing a lavage rinse containing 25 mg. of citric acid

TABLE 1. PERITONEAL WASH ANALYSIS OF CALCIUM AND CITRATE IN NEPHRECTOMIZED AND NEPHRECTOMIZED-PARATHYROIDECTOMIZED RATS

All values given in mg./100 ± S.E. $\left(\pm\sqrt{\frac{\sum d^2}{n(n-1)}}\right)$

Amt. of citrate in rinse	Time (hours)	Exp. group	No. of animals	Period											
				1		2		3		4		5		8	
				Ca	Cit	Ca	Cit	Ca	Cit	Ca	Cit	Ca	Cit	Ca	Cit
15.0 mg.	1½	N	18	4.5 ± .13	0.82 ± .19	5.4 ± .16	3.8 ± .17	6.1 ± .15	3.5 ± .18	6.4 ± .14	2.9 ± .17	6.6 ± .17	2.5 ± .20		
		N-PTX	14	3.0 ± .12	0.57 ± .15	3.7 ± .11	3.4 ± .18	4.2 ± .15	3.3 ± .18	4.9 ± .13	2.9 ± .21	5.1 ± .17	2.6 ± .15		
25.0 mg.	1	N	11	4.4 ± .10	0.74 ± .24	5.4 ± .10	8.9 ± .29	5.7 ± .18	8.9 ± .39	5.7 ± .16	8.0 ± .39	6.1 ± .15	7.4 ± .31	6.3 ± .10	6.6 ± .51
		N-PTX	8	2.7 ± .20	0.47 ± .31	3.3 ± .23	8.9 ± .24	3.5 ± .14	8.5 ± .34	3.7 ± .10	8.4 ± .34	3.9 ± .12	7.8 ± .17	4.4 ± .17	6.2 ± .23
25.0 mg.	1½	N	19	4.9 ± .11	0.78 ± .15	6.1 ± .13	5.5 ± .36	6.5 ± .12	5.7 ± .33	7.0 ± .16	5.2 ± .32	7.3 ± .15	4.7 ± .20		
		N-PTX	14	3.2 ± .13	0.54 ± .12	4.0 ± .11	4.9 ± .32	4.5 ± .13	5.2 ± .28	4.8 ± .10	4.9 ± .28	5.2 ± .10	4.2 ± .28		

per 100 ml. and a rinse equilibration time of 1½ hours:

Equilibration in the peritoneal cavity for an additional half-hour caused a significant increase in the amount of calcium removed in both control and parathyroidectomized animals, and also a reduction to physiological levels of the amount of citric acid remaining in the wash. These data are also tabulated in Table 1.

B. *Effects of citric acid on the radiocalcium removed by continuous peritoneal lavage*

1. Experiments in which the radiocalcium was injected 18 hours prior to peritoneal lavage:

For all these studies on the removal of radiocalcium from bone as influenced by citric acid, equilibration periods of one and one-half hours were used, and citric acid (25 mg./100 cc.), when employed, was added to all rinses but the first. Since the animals had been parathyroidectomized the day prior to the experimental treatment, the calcium levels of the first wash, which was citrate-free, were already at the reduced levels previously reported resulting from parathyroidectomy (16). The calcium levels in each successive wash rose as reported in the experiments described above. The actual radioactivity removed remained essentially unchanged by either parathyroidectomy or citric acid addition. As a result, the specific activities for both control and parathyroidectomized groups receiving citrate declined rapidly. These changes are illustrated in Figure 1, and the data from these experiments are tabulated in Table 2. It can only be concluded that the exogenous citric acid did not take calcium from those areas which contained radiocalcium during the first twenty-four hours after the injection of the radioactivity.

One group of rats was rinsed with a fluid containing a lethal dose of citric acid. One rinse period was completed prior to the death of the animals. While total radioactivity of the wash showed only a slight drop (1000 to 890), the specific activity declined to 43% of its citrate-free wash value, due to a citrate-induced calcium level of 9.6 mg. per 100 ml.

2. Experiments in which the radiocalcium was injected two hours prior to peritoneal lavage:

Several experiments were run similar to those described above, except that the radioactivity was administered only two hours prior to the starting of the continuous lavage. These are summarized in Table 3. Because of the short time in which the radioactivity had been in the animals, there was a marked decline in the amount of radiocalcium removed in successive periods in the control animals. However, similar curves of removal of radioactivity were also seen in the experimental groups. While the declines in total activities were reflected in rapid drops in the specific activities for all rats, animals receiving citrate rinses were seen to drop significantly faster than groups receiving no citrate.

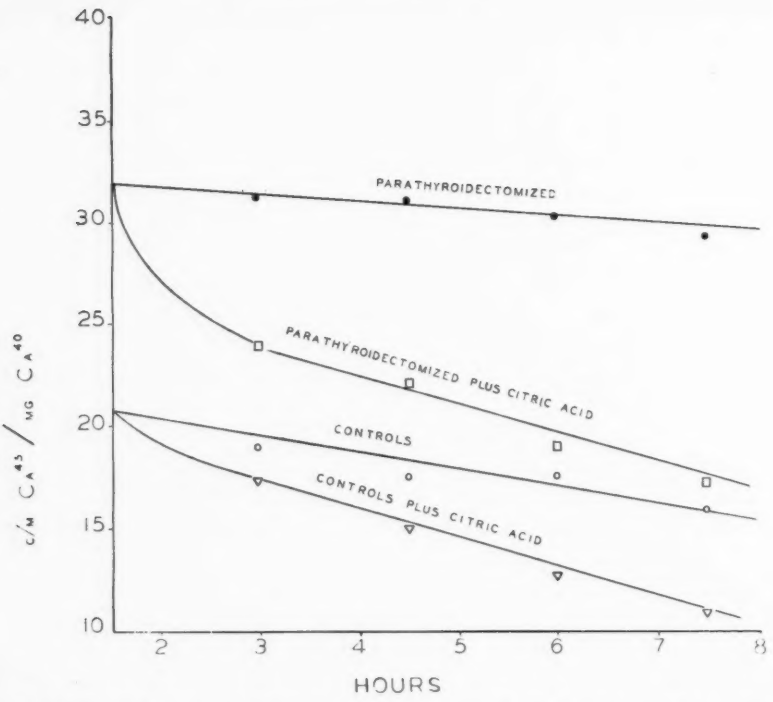

FIG. 1. Effect of exogenous citric acid on specific activity of Ca^{45} removed by peritoneal lavage (Ca^{45} administered 18 hours previous to lavage). Specific Activity = Counts per minute of Ca^{45} divided by ml. Ca × 1000. See Table 2 for other data.

TABLE 2. EFFECT OF CITRIC ACID ON THE REMOVAL OF Ca^{40} AND Ca^{45} BY CONTINUOUS PERITONEAL LAVAGE
(Radioactivity injected 18 hours prior to lavage)

	Exp. condition	Number of animals	Period				
			1	2	3	4	5
Radioactivity Ca^{45} in C/min. Ca^{45}/ml.	Neph	8	1000 ± 29	886 ± 24	863 ± 28	862 ± 28	839 ± 29
	Neph & Cit	7	993 ± 43	948 ± 37	919 ± 40	907 ± 38	892 ± 34
	PTX-Neph	9	1047 ± 97	1068 ± 79	1060 ± 93	1045 ± 70	1063 ± 58
	PTX-Neph & Cit	7	1013 ± 49	973 ± 41	984 ± 47	1011 ± 39	993 ± 42
Specific Activity in C/min. Ca^{45}/mg. Ca	Neph	8	207 ± 4	188 ± 7	173 ± 6	179 ± 5	173 ± 6
	Neph & Cit	7	213 ± 5	177 ± 8	151 ± 4	128 ± 5	113 ± 6
	PTX-Neph	9	342 ± 18	333 ± 21	330 ± 23	312 ± 24	307 ± 17
	PTX-Neph & Cit	7	301 ± 14	235 ± 9	214 ± 9	186 ± 11	166 ± 7
Total Calcium, mg. Ca/ml. × 100	Neph	8	4.8 ± .16	4.7 ± .13	5.0 ± .18	4.8 ± 1.5	4.8 ± .18
	Neph & Cit	7	4.6 ± .13	5.4 ± .19	6.1 ± .21	7.1 ± .24	7.9 ± .27
	PTX-Neph	9	3.0 ± .11	3.2 ± .16	3.2 ± .14	3.3 ± .16	3.5 ± .18
	PTX-Neph & Cit	7	3.3 ± .13	4.1 ± .17	4.6 ± .20	5.4 ± .22	6.0 ± .23

Notes: 1) Citric acid (25 mg./100 ml.) added to rinse beginning with second lavage period.
2) Radioactivity removed in the first wash of nephrectomized control group set at 1000. All other values adjusted to this standard.
3) Values given with standard error = $\pm \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

TABLE 3. EFFECT OF CITRIC ACID ON THE REMOVAL OF Ca^{45} BY CONTINUOUS PERITONEAL LAVAGE

(Radioactivity injected two hours prior to rinsing)

	Exp. condition	Number of animals	Period				
			1	2	3	4	5
Radioactivity Ca^{45} in C/min. Ca^{45}/ml.	Neph	5	1000 ± 30	676 ± 34	596 ± 37	540 ± 29	447 ± 22
	Neph & Cit	5	996 ± 32	740 ± 38	638 ± 26	554 ± 29	463 ± 24
	PTX-Neph	4	1013 ± 42	726 ± 36	575 ± 42	519 ± 27	461 ± 24
	PTX-Neph & Cit	4	1048 ± 29	704 ± 34	591 ± 31	507 ± 28	433 ± 30
Specific Activity C/min. Ca^{45}/mg. Ca.	Neph	5	217 ± 5	119 ± 6	117 ± 6	106 ± 7	96 ± 4
	Neph & Cit	5	226 ± 9	134 ± 7	91 ± 7	76 ± 5	61 ± 5
	PTX-Neph	4	307 ± 11	220 ± 8	165 ± 7	144 ± 5	128 ± 6
	PTX-Neph & Cit	4	318 ± 13	156 ± 10	123 ± 6	89 ± 7	73 ± 6

Notes: 1) Citric acid (25 mg./100 ml.) added to rinse beginning with second lavage period.
2) Radioactivity removed in the first wash of nephrectomized control group set at 1000. All other values adjusted to this standard.
3) Values given with standard error = $\pm \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

DISCUSSION

These studies are considered as additional evidence of the close relationship of citric acid, parathyroid hormone and calcium metabolism. They clearly demonstrate that near-physiological levels of citric acid administered by peritoneal lavage are capable of mobilizing calcium both in the presence and absence of endogenous secretion of parathyroid hormone. That this mobilization is from bone, and not from extra-osseous sources of calcium, is indicated by the results showing that the calcium is taken from areas which are not in equilibrium with recently injected radiocalcium. It seems of interest, also, that these raised levels of calcium were achieved and maintained after the citric acid levels had fallen to within normal physiological ranges.

Recent work from this laboratory (18) has suggested that endogenous parathyroid hormone mobilizes calcium from the deeper areas of bone, namely, those areas which do not come into contact with radiocalcium during the first twenty-four hours after its administration. The mobilization of calcium by exogenous citric acid from the same areas of bone is indicated by these experiments. This study, therefore, points again to the relationship of the parathyroids and citric acid, but it also raises the question as to why the acid is unable to take calcium from those areas in closer contact with the extracellular fluid media. One would be tempted to suggest that citric acid must be carried to areas of bone where it becomes a part of an undetermined metabolic sequence through which it mobilizes calcium. While calcium can be removed in the absence of the hormone of the parathyroids, the acid does not by any means completely replace the hormone, suggesting that both work through a more complex metabolic process. It is hoped that further study will help elucidate these points. Thus, more evidence has been presented which indicates that citric acid or some similar acid may be the agent in bone under the control of the parathyroid

hormone which is normally involved in the metabolic processes causing r[illegible]-absorption of calcium from bone.

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REMOVAL OF CALCIUM FROM BONE AS INFLUENCED BY THE PARATHYROIDS[1]

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ABSTRACT

This report is a study of the relative removal of radiocalcium and stable calcium by continuous peritoneal lavage in the rat, as influenced by the hormone of the parathyroids. The lavage procedure involved the use of male rats weighing between 225 and 275 gm. which had been maintained 48 hours on a calcium free diet and had been nephrectomized 24 hours before experimental use. Parathyroidectomy was performed without interruption of the lavage procedure. The results indicated that if the radiocalcium was injected into the animals less than 24 hours before the lavage treatment, the radioactivity removed by this method was not affected by parathyroidectomy. This was in spite of the fact that the operation caused an immediate drop in the total calcium concentrations of the lavage washes. If, however, the radiocalcium was injected two to three weeks before the continuous lavage was performed, the radiocalcium concentrations of the wash parallel those of the stable calcium, both falling after parathyroidectomy.

These data are construed to indicate that a basic equilibrium exists between the extracellular spaces and those portions of the bone with which the fluid is in ready contact, and that this equilibrium maintains the constant low concentration of calcium in the fluid spaces. It is further postulated that, in addition to this basic equilibrium, parathyroid hormone supplies calcium to the fluid spaces from areas of bone that are not in immediate contact with the surrounding fluid media, and that this is done through metabolic processes in bone cells which enables calcium to be dissolved in concentrations greater than the solubility of apatite crystals.

THE ability of bone to supply calcium continuously to the extracellular spaces has been studied recently by Copp (1) and noted in reports from this laboratory (2, 3). Earlier, McLean and Urist (4) had suggested that the action of parathyroid hormone on bone was on the order of a "feed-back" mechanism. In their concept, the hormone, through its control on bone dissolution, was able to raise blood calcium concentrations above the basic level maintained in its absence. Work from this laboratory (3) has shown, by the use of continuous peritoneal lavage, that bone is capable of immediately supplying large amounts of calcium to the extracellular spaces both in the intact and the parathyroidectomized rat. However, the equilibration levels in the peritoneal wash of the operated animal was ap-

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[1] Aided in part by a grant from the Atomic Energy Commission. Some of these results were reported at the UNESCO Conference on the Use of Radioisotopes in Scientific Research, Paris, September, 1957.

proximately two-thirds that of its parathyroid-intact control. It was suggested that the basic ability of bone to supply calcium might be primarily a physical process, while the higher levels maintained by the parathyroids were accomplished through the aid of citric acid production by bone cell components. The connection of citric acid to the function of the parathyroids has been stressed recently in reports by Freeman and Elliott (5, 6), Neuman *et al.* (7) and by Elliott and Talmage (8, 9).

The present studies were carried out with the hope that, through the use of radiocalcium, further differences between the processes by which bone released calcium, in the presence and absence of the parathyroids, could be elucidated. Comar, in a series of papers utilizing both laboratory and farm animals (10), has followed the incorporation of both radiocalcium and radiophosphorus into long bone from its immediate deposition or chemical exchange into the subepiphyseal and periosteal regions to its gradual incorporation into the deeper bone trabeculae by the process of bone remodelling. Based on this progressive change in the distribution of radioactivity over a period of time, the following studies were carried out by utilizing the technique of peritoneal lavage in animals to which radiocalcium had been administered either between twelve and twenty-four hours or three weeks prior to their experimental use. These experiments suggest that bone utilizes two different mechanisms in supplying calcium to extracellular compartments and indicate that under different physiological conditions calcium is withdrawn from different localities in the bone.

MATERIALS AND METHODS

Male Holtzman rats, weighing between 225 and 275 gm., were used in these experiments. Peritoneal lavage was accomplished by indwelling inguinal catheters as previously described (3, 11). The lavage rinse consisted of .84% NaCl plus 1.5% glucose, maintained at 40° C in a water bath. (In the discussions to follow the word "rinse" is used to refer to the fluid placed in the animal; the word "wash" to refer to the fluid removed from the peritoneal cavity.) The animals were kept under light nembutal anesthesia throughout the experimental period. This was administered with the rinse solution in amounts averaging 2 mg. of sodium pentobarbital per hour per animal. Calcium determinations were made by a procedure previously described (8) involving precipitating the calcium with ammonium oxalate, redissolving in perchloric acid and reading on a Beckman flame spectrophotometer. Radioactive calcium (Ca^{45}) was administered in an essentially carrier-free form by intraperitoneal injection. The animals were injected with the isotope either eighteen to twenty-four hours or two to three weeks prior to their experimental use. Radioactive determinations were made on aliquots of the wash solution dried down in weighed stainless steel cups, and counted in an automatic flow counter with subsequent adjustments for self-absorption. The amount of activity injected varied from 10 to 50 μc. However, all animals in any single experiment received identical amounts of the isotope. For purposes of this paper, specific activity is defined as the relative amount of radioactivity (Ca^{45}) per unit weight of the total element (Ca^{40} plus Ca^{45}) present.

Experimental procedure: For each experiment, the following basic pattern was used:

Animals previously maintained on a calcium-deficient diet[2] for twenty-four hours were bilaterally nephrectomized. The morning after nephrectomy, catheters were inserted through the inguinal canals, and the peritoneal lavage commenced. For these rinses, 30 ml. of the fluid was placed in each animal and immediately removed. A second 30 ml. was placed in the cavity and allowed to equilibrate for either one or one and one-half hours, depending upon the needs of the experiment. The wash was then removed for analysis, and an additional 30 ml. was introduced into the animal for the succeeding equilibration period. This procedure was continued throughout the experiment which consisted of from 5 to 8 successive washes. (Parathyroidectomy, where performed, was done during the third lavage period without interruption of the lavage schedule.) In every experiment, both nephrectomized- and nephrectomized-parathyroidectomized rats were used.

RESULTS

A. *Removal of Ca^{45} injected 18 to 24 hours prior to lavage.* For these experiments, nephrectomy was followed 6 to 8 hours later by the injection of Ca^{45}. Continuous peritoneal lavage was commenced the following morning, and parathyroidectomy was performed during the third lavage period. The data from these experiments are summarized in Figure 1. For simplicity of presentation and in order to compare the various experiments statistically, the radioactivity data were adjusted in the following manner: Since each animal served as its own control, the average value for the radioactivity of the first wash of each experiment was set at 1000. The actual radioactive measurements determined for all washes were then multiplied by the factor required to correct the first wash value to 1000.

The changes in the calcium levels of the successive washes followed the pattern reported previously (3). For one hour equilibration periods, the control values averaged .045 mg. per ml. of wash. Following parathyroidectomy, the calcium values fell to .033 mg. per ml. and remained fairly constant throughout the successive washes. The radioactivity of the wash did not, however, follow this pattern, both control and experimental values dropped gradually, but together. As a result, the specific activity of the radiocalcium rose, following removal of the parathyroids, to values approximately 1.5 times that of the control values.

B. *Removal of Ca^{45} injected 2 to 3 weeks prior to lavage.* Several experiments were also run in which the radiocalcium was injected sufficiently long before the experimental use of the animals to permit a deeper and more general penetration of the radioactivity throughout the bone. The accumulated data from these experiments are summarized in Table 1. In contrast to the experiments summarized above in which parathyroidectomy had no effect on the rate of removal of radiocalcium, in these experiments the radioactivity values diminished in proportion to the fall in total

[2] The composition of the calcium deficient diet was as follows: Alphacel—23.9%, Dextrose—21.7%, Sucrose—8.7%, Lard—17.4%, Wesson Oil—2.6%, Cod Liver Oil—0.6%, Corn Starch—25.1%.

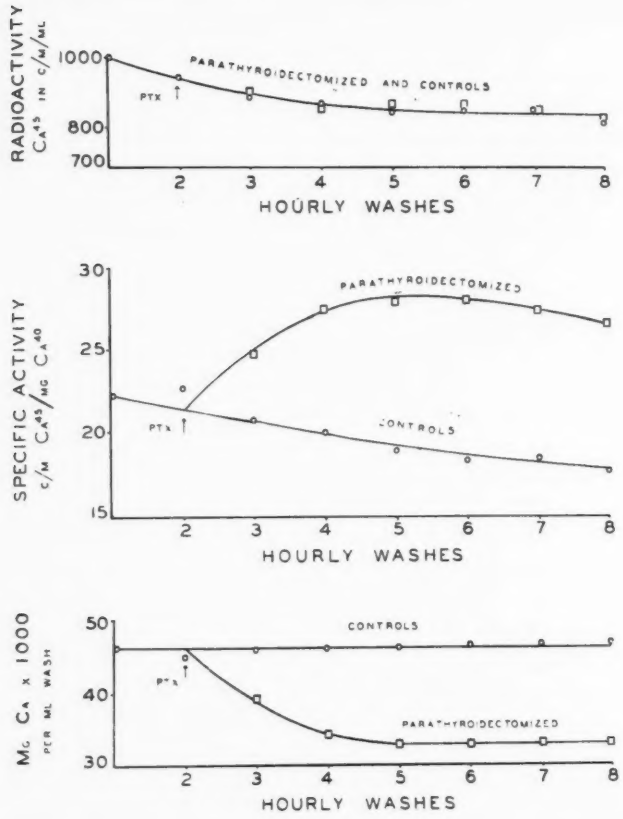

FIG. 1. Effect of parathyroidectomy on the removal of Ca^{45} and Ca^{40} by continuous peritoneal lavage (radioactivity injected 18 hrs prior to lavage).

Each point on the graph represents the average value of 12 animals. Standard Error was determined by the formula

$$\pm \sqrt{\frac{\sum d^2}{n(n-1)}}$$

For the top two graphs, the average standard error was 5% of the value; for the bottom graph, the error was 2%. PTX↑ indicates the time parathyroidectomy was performed.

calcium. Thus, parathyroidectomy produced no changes in specific activity values.

DISCUSSION

The interpretation of these data is based on the well-known fact that, following a single injection of radiocalcium, radioactivity is first concentrated in specific areas of bone, particularly in the epiphyseal regions of

long bones. Only gradually does the radioactive calcium penetrate into the deeper bone structures. This is accomplished presumably by the remodelling processes continuously being carried on in the bone, aided in addition by the gradual process of simple chemical exchange. Therefore it is a safe assumption that, during the first twenty-four hours, most of the activity is still concentrated in those parts of the bone which are in closer contact with the fluid media of the extracellular spaces. If parathyroidectomy is performed during the period, the radioactivity removed by lavage remained remarkably constant despite the marked drop in total calcium concentra-

TABLE 1. THE EFFECT OF PARATHYROIDECTOMY ON THE REMOVAL OF Ca^{45} AND Ca^{40} BY CONTINUOUS PERITONEAL LAVAGE
(Radioactivity administered 2–3 weeks previously)

	No. of animals	Total No. of washes	Radioactivity*	Mg. Ca/ml. × 1000	Specific activity†
Controls	6	20	1000 ± 33.5	48.5 ± 2.0	20.9 ± 1.5
Parathyroidectomized	4	18	675 ± 10.4	32.0 ± 0.9	21.0 ± 0.6

* For comparative purposes, the average radioactivity value of the controls (Ca^{45} in c./m./ml.) for each wash was set at 1000, and all values for that wash adjusted to this standard. Each value represents the average of all washes and is given with

† Specific Activity $= \frac{\text{c./m./ml. } Ca^{45}}{\text{mg./ml. } Ca^{40} + Ca^{45}}$.

tions of the washes. However, by leaving the radioactivity in the animal sufficiently long to allow for a more even distribution throughout the bone, the removal of radioactivity then followed closely the removal of stable calcium.

These data would seem to make it valid to conclude that the basic ability of bone to supply calcium to the extracellular fluid spaces, such as is seen in the parathyroidectomized animal, could easily be an equilibrium phenomenon between the fluid compartments and those portions of bone with which the fluid is in most direct contact. As suggested before (3), this equilibrium could be based primarily on physical processes, namely the solubility of calcium phosphate salts such as apatite crystals in isotonic saline. However, whether the speed with which these ions are supplied "in vivo" can be explained entirely on the basis of physical dissolution is open to considerable question.

On the other hand, the site of action of the parathyroid must be on the deeper bone trabeculae, and not the most recently deposited calcium. This is indicated by the observation that the radiocalcium had not reached the area affected by the parathyroids by the end of twenty-four hours after its injection. Evidence is accumulating to indicate that this is an active metabolic process, closely concerned with one or more of the cellular components

of bones in a process which supplies calcium to the fluid media in concentrations greater than the solubility of apatite crystals at the pH of physiological media. The recent reports on the close connection of citric acid and parathyroid function make it seem worthwhile to repostulate that it could easily be through the acidity and calcium-complexing ability of citric acid that parathyroid hormone achieves its ability to maintain and supply the high levels of calcium in the serum.

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THE EFFECT OF EPINEPHRINE AND NOR-EPINEPHRINE ON THE ACUTE THYROID RELEASE OF THYROID HORMONES

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ABSTRACT

Catheterization of the thyroid vein and measurement of its PBI^{131} levels have been utilized to study the effect of epinephrine and nor-epinephrine on the release of thyroid hormones from the thyroid in dogs. After intravenous administration of epinephrine to seven dogs and of nor-epinephrine to three dogs, increases in PBI^{131} levels from 1.5 to 17.1 times the control values were found. These responses occurred within 15 to 95 minutes in all dogs tested. Control experiments in three dogs have shown that during a comparable experimental period of four hours, no significant change in thyroid venous PBI^{131} concentrations took place.

In two dogs given thyroid stimulating hormone (TSH) intravenously, increases in thyroid venous PBI^{131} levels of 18.9 and 7.4 times the control activities were noted. The initial responses occurred within 20 minutes after starting the TSH infusion. In two hypophysectomized dogs, epinephrine infusion was attended with a rise in thyroid venous PBI^{131} comparable to that seen in the intact animal. These data would indicate that the stimulating effect of epinephrine is exerted directly on the thyroid gland.

Preliminary radiopaperchromatographic studies in one dog revealed that the increase of PBI^{131} observed in the thyroid vein during epinephrine stimulation was attributable to rises in both thyroxine and triiodothyronine. The possible participation of increased hormonal secretion from the thyroid gland in the acute metabolic response to stress has been discussed in the light of these data.

INTRODUCTION

THE demonstration of 3:5:3′-L-triiodothyronine in human serum (1, 2) and the potent and rapid physiologic action of this substance in animals (3, 4, 5) and man (6, 7, 8, 9) has necessitated a re-appraisal of the role of the thyroid gland in the acute metabolic response of the body to stress. The thyroid gland has been looked upon traditionally as a sluggishly functioning organ since the only hormone it was formerly known to produce, thyroxine, requires some two to three days to produce a measurable effect on oxygen consumption in man (10, 11). Because of the delayed physiologic response to thyroxine, this hormone has been thought to play an insignificant role in the acute body reaction to stress. Triiodothyronine, on the

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other hand, produces definite metabolic changes in man within four to 12 hours (8, 9) and could conceivably serve as an "emergency" type of thyroid hormone released in times of stress.

It has also been postulated by many investigators that epinephrine released from the adrenal medulla may serve as an important intermediary in the body response to stress by activating the anterior pituitary which, in turn, results in increased adrenocortical (12, 13, 14, 15) and thyroid activity (16, 17, 18). For this reason a study of the effect of epinephrine on the release of thyroid hormones from the thyroid gland of dogs was undertaken.

Experimental animals were first administered large tracer doses of inorganic I^{131}. After an interval of 48 to 72 hours to permit I^{131} labeling of the hormones in the thyroid gland, the effect of epinephrine, nor-epinephrine and thyroid stimulating hormone on the levels of the protein bound iodine^{131} (PBI^{131}) in the thyroid veins of these animals was determined. It was felt that the blood taken directly from the thyroid vein before it mixed with the remainder of the intravascular volume would reflect more accurately small changes in the release of thyroid hormone than would peripheral blood samples. Radiopaperchromatographic techniques were utilized in some studies in order to determine which of the hormones released from the thyroid gland showed demonstrable changes in their secretory levels during the experimental period. Two experiments were also carried out in hypophysectomized dogs to help elucidate the pathway of epinephrine action.

MATERIALS AND METHODS

The experimental animals consisted of male and female dogs weighing 10 to 15 kg. and maintained on a diet of low iodine content. One hundred to 500 microcuries of carrier-free NaI^{131} obtained from the Oak Ridge National Laboratories were injected intravenously into these animals approximately 48 to 72 hours before the experimental periods.

Surgical Procedures

Cannulization of Thyroid Vein. A technique for collecting blood from the relatively minute thyroid veins of the dog was developed. Under nembutal anesthesia a midline incision was made in the neck from the thyroid cartilage down to several centimeters above the sternum. The thyroid lobes and their vessels were then dissected out and a fine polyethylene catheter of 0.86 mm. internal diameter was inserted into one of the inferior or superior thyroid veins. The other thyroid vessels remained undisturbed. A catheter was placed in the femoral artery also and connected to a mercury manometer in order to measure mean arterial blood pressure. Three mg. of heparin per kg. were infused intravenously into each animal in order to avoid clotting within the catheters. To help maintain blood volume throughout the experiment, a slow intravenous infusion of 5 per cent dextrose in physiological saline solution was administered.

Hypophysectomy. A transbuccal approach was employed in this procedure. Under nembutal anesthesia the soft palate was incised and the edges of the mucosa were retracted laterally. The mucoperiosteum was then incised in the midline and freed from

the underlying bone. Using a small dental burr and rongeurs, an opening was made in the bone between the intersphenoid and the occipitosphenoid sutures. The dura was then opened, and the pituitary gland removed *in toto*. The incisions were closed and the animals were given prophylactic penicillin (300,000 units) and streptomycin (0.5 gram).

On completion of the entire experiment, the animals were sacrificed and the tissue in the pituitary fossa was carefully removed to curette for histological study. No visible pituitary cells were found in either of the two animals utilized in this study, and both were therefore considered to have had total hypophysectomies.

Experimental Procedures

The effect of epinephrine, nor-epinephrine and thyroid stimulating hormone on the PBI^{131} plasma levels was investigated. At intervals of 10 to 30 minutes during the control and experimental infusion periods, and for 30 to 200 minutes after infusion, serial samples of blood (5 ml.) were collected in potassium oxalate tubes from the indwelling catheters of the femoral artery and thyroid vein. A total of 20 to 30 blood samples was usually drawn. The duration of the control period varied from 60 to 120 minutes, after which the substance under study was introduced into the saphenous vein.

Epinephrine was administered in a 5% dextrose-in-saline solution to seven dogs and was given at a rate sufficient to raise the mean blood pressure about 30 mm. of mercury. This was maintained for a period of 60 to 210 minutes. Nor-epinephrine was administered in a similar manner to three dogs for 140 to 195 minutes. The rise in blood pressure was again used as a guide for the rate of infusion. The actual quantities of the two drugs administered varied from one to three micrograms per kg. per minute, with mean total doses of approximately 130 to 400 μg. per kg. to each dog. Ten to 20 units of thyroid stimulating hormone (Thytropar Armour[2]) in 100 ml. of physiologic saline solution were given intravenously to two dogs during a period of 65 minutes.

Three control experiments were carried out by collecting samples from the thyroid vein and femoral artery for 150 to 240 minutes under circumstances identical with the experimental conditions, except for the omission of the test substances from the 5% dextrose-in-saline infusion.

Additional experiments to investigate the pathway of epinephrine action were carried out on two hypophysectomized dogs. In these animals the tracer doses of I^{131} had been administered 24 to 48 hours prior to pituitary ablation to allow for intrathyroid labeling of thyroid hormones. The conditions of these experiments were otherwise identical to those employed in the epinephrine studies on the intact animals. Approximately 24 hours after hypophysectomy equivalent doses of epinephrine were administered over periods of 110 and 145 minutes, and the usual samples were collected from the thyroid vein and the femoral artery. Hypophysectomy effectively prevents any new secretion of TSH following epinephrine but, of course, does not rule out the possibility that any effect of epinephrine on the thyroid gland might require not increased TSH secretion but continued action of TSH at the thyroid level. Only 24 hours after hypophysectomy some activity of TSH could still be expected.

Measurement of Radioactive Protein Bound Iodine

Samples from the thyroid vein and femoral artery were centrifuged and 2.0 ml. of plasma drawn off. The radioactivity of the plasma was first measured in a well type scintillation counter by counting to a total of 5,000 counts, and represented the total plasma radioactive iodine (inorganic and organic iodine). The method of Silver *et al.* (19) was utilized for the determination of the protein bound radioactive iodine. Four

[2] The thyrotropin was supplied through the courtesy of the Research Division of the Armour Company, Kankakee, Illinois.

ml. of a 10% solution of trichloroacetic acid were used to precipitate the protein bound radioactive iodine of the 2.0 ml. of plasma, and the precipitate was then washed three times with 4.0 ml. of the trichloroacetic acid solution. The precipitate was then redissolved with 1.5 ml. of 4N sodium hydroxide. This sample was made up to volume and its activity determined in a well type scintillation counter by counting to a total of 5,000 counts. This measurement represented the radioactive protein bound iodine (PBI^{131}) in 2.0 ml. of plasma.

Radiopaperchromatographic Technique

Radiopaperchromatographic studies of plasma samples were carried out in a few experiments by a technique similar to that described by Benua and Dobyns (20). Approximately 3.0 ml. of plasma were first slurried with 0.5 gm. (dry weight) of an anion exchange resin (Amberlite IRA-400) to remove excesses of inorganic I^{131}, which occasionally tended to obscure the contour of the more important adjacent activity peaks. The plasma was then acidified to pH2 with 5N HCl, extracted three times with normal butanol, and evaporated to dryness in a 50° C water bath. The residue was then taken up in a few drops of ethanol—27% ammonium hydroxide (9:1) and after the addition of approximately 10 μg. of thyroxine, triiodothyronine,[3] and diiodotyrosine carriers, it was placed on Whatman #1 filter paper in a butanol-dioxane-2N ammonium hydroxide (4:1:5) ascending solvent system for 24 hours. The site of the carriers was determined by spraying with diazotized sulfanilic acid.

The paperchromatogram was then cut into 0.5 cm. horizontal strips for radioactivity measurement in a well type scintillation counter, each strip being counted for one minute. In this fashion, the relative amounts of radioactivity corresponding to the known areas on the chromatogram could be determined.

RESULTS

Control Period

Radioactivity measurements of plasma samples from the thyroid vein and femoral artery during control periods showed a similar pattern in all of the experiments. The *total* plasma radioactive iodine levels were slightly higher in the blood taken from the femoral artery. This finding reflects the ability of the thyroid gland to trap inorganic iodide from the circulating arterial blood. On the other hand, the thyroid venous PBI^{131} levels were usually significantly higher than the corresponding arterial levels, the result of a continuous release of small quantities of protein bound iodine from the gland. PBI^{131} levels during the control period remained relatively constant in the samples from the femoral artery and the thyroid vein in each of the experiments (Figs. 1, 2, 3, 4, 5).

Control Experiments

Three control experiments were carried out over a period of three to four hours, during which time PBI^{131} values from the thyroid vein and femoral artery were measured. Experimental conditions were identical with those involving epinephrine and nor-epinephrine, except that saline alone was in-

[3] Thyroxine and triiodothyronine carrier materials for the chromatographic studies were furnished by the Smith, Kline and French Laboratories, Philadelphia, Pennsylvania.

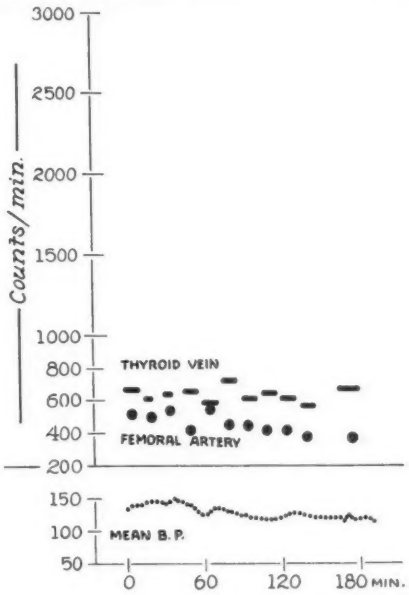

FIG. 1. No significant changes noted over a three-hour period of saline infusion. Conditions were identical to those in the experiments employing epinephrine and nor-epinephrine.

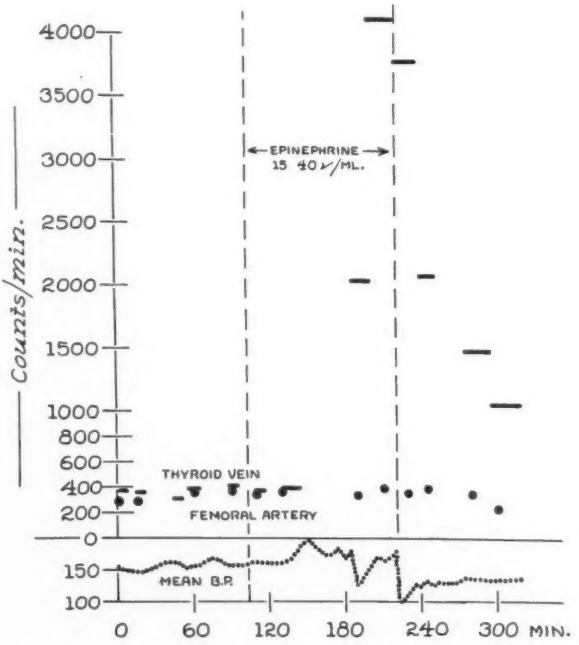

FIG. 2. (Dog 6.) The ratio of the peak PBI^{131} level during the infusion period to the control levels was 10.3. The response of the thyroid venous PBI^{131} to epinephrine was somewhat less prompt than in other studies with epinephrine.

fused. No significant change was noted in the PBI^{131} of either the thyroid vein or the femoral artery (Fig. 1).

Epinephrine Administration

Comparable results were obtained in all seven epinephrine experiments. After the start of the epinephrine infusion, there was a latent period of 15 to 95 minutes during which the thyroid venous PBI^{131} concentrations re-

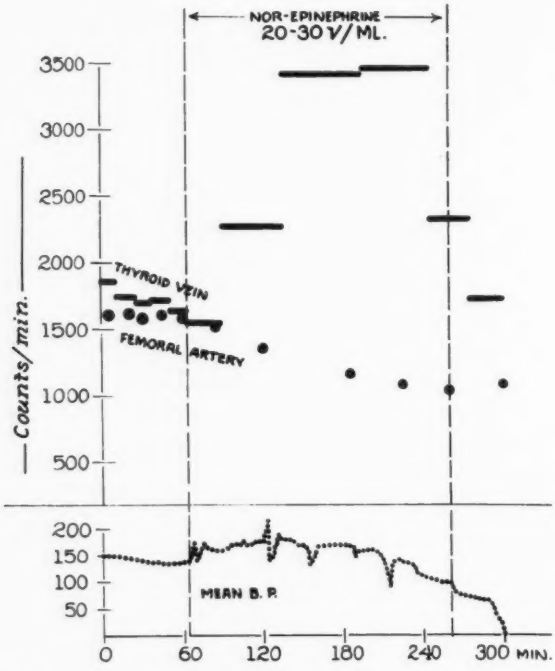

FIG. 3. (Dog 10.) The ratio of the peak PBI^{131} level during the infusion period to the control values was 2.0.

mained at control values. Following this period the PBI^{131} levels in the thyroid vein rose sharply and reached a peak usually just before or soon after the epinephrine was discontinued (Fig. 2). The ratios of these highest levels to the control levels varied from 1.5 to 10.3 in the seven experiments, with a mean ratio value of 4.8. These results are summarized in Table 1. The lowest ratio occurred in the initial experiment when the procedure was not completely perfected and when the epinephrine was administered in the smallest quantity for the shortest period of time. A sharp decline in the PBI^{131} levels usually occurred immediately after stopping the epinephrine infusion. The levels of PBI^{131} in the femoral artery did not show an accom-

paying rise in any experiment during the infusion of epinephrine but instead remained the same or decreased slightly.

Since PBI^{131} *concentrations* were measured in the samples collected from the thyroid vein rather than the *total* PBI^{131} activity passing through the catheterized vein during the control and experimental periods, it is pertinent to point out that measurements of blood flow in the thyroid vein were not made during the experiments reported in this study. It is conceivable

TABLE 1. SUMMARY OF THE EFFECTS OF THE INFUSION OF EPINEPHRINE, NOR-EPINEPHRINE AND THYROID STIMULATING HORMONE ON THYROID VENOUS PBI^{131}

Dog	Drug administered	Duration of infusion of drug	Time of 1st PBI^{131} increase	Time of peak PBI^{131} increase	Ratio of peak PBI^{131} level to control PBI^{131} level
1	Epinephrine	60′	15–40′	15–40′	1.5
2	Epinephrine	170′	35–50′	170–200′	7.9
3	Epinephrine	210′	95–125′	175–210′	2.5
4	Epinephrine	110′	40–60′	90–110′	4.2
5	Epinephrine	115′	0–35′	70–110′	3.2
6	Epinephrine	120′	85–110′	100–120′	10.3
7	Epinephrine	90′	60–70′	120–200′	4.2
8	Nor-epinephrine	140′	60–85′	200–240′	17.1
9	Nor-epinephrine	175′	85–110′	175–205′	2.3
10	Nor-epinephrine	195′	25–70′	130–180′	2.0
11	TSH	65′	15–35′	115–135′	18.9
12	TSH	65′	20–45′	220–250′	7.4
13	Epinephrine (Hypox. dog)	110′	80–95′	95–110′	2.5
14	Epinephrine (Hypox. dog)	145′	35–80′	145–160′	3.2

that changes in thyroid venous blood flow alone induced by the administration of epinephrine could produce rises in the thyroid venous PBI^{131} values as were noted during the infusions of epinephrine. It is unlikely, however, that changes in blood flow per se significantly affected the thyroid venous PBI^{121} values reported for the following reasons.

During the control periods spontaneous, wide variations often occurred in the thyroid venous flow, as judged by the time necessary to collect the venous samples, without significant changes taking place in the PBI^{131} concentrations. Also, it was possible in some experiments to calculate the total number of radioactive counts passing through the thyroid vein, both before and during the infusion of epinephrine, because the flow was so slow that a summation of the individual venous samples equaled the total flow through the vein. In these instances significant increases in the total PBI^{131} counts were also found during the epinephrine infusions comparable to the increases noted in the PBI^{131} concentrations. Finally, it would seem improbable that increases in thyroid venous PBI^{131} concentrations as high as 10 times the control levels would occur on the basis of changes in blood flow alone, and are better explained on the basis of an increased release of thyroid hormone from the gland. Thus, it would appear that variations in

thyroid venous blood flow did not play a significant role in the changes in the PBI^{131} concentration values reported during the epinephrine and other infusions utilized in this study.

Nor-epinephrine Administration

In three experiments, after a latent period of 25 to 85 minutes, administration of nor-epinephrine produced rises of PBI^{131} levels in the thyroid

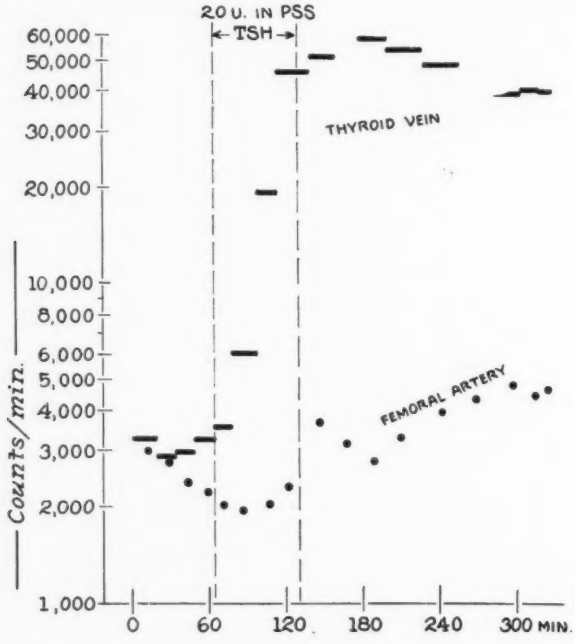

FIG. 4. (Dog 11.) The ratio of the peak PBI^{131} level during the infusion to the control levels was 18.9. The persistence of the elevated PBI^{131} even after TSH was discontinued is striking.

vein similar to those observed during the epinephrine infusions (Fig. 3). The ratio of these levels at their peaks to the control values ranged from 2.0 to 17.1 in all instances. Arterial levels of PBI^{131} again showed no change. A summary of these experiments is shown in Table 1.

Thyroid Stimulating Hormone Administration

In two experiments the infusion of thyroid stimulating hormone (TSH) produced results grossly similar to those obtained with epinephrine and nor-epinephrine. The responses by the thyroid gland were very rapid in both instances. Initial rises in PBI^{131} levels of the thyroid vein occurred at

15 and 20 minutes respectively immediately following TSH administration (Table 1). The levels did not fall appreciably after the infusions were stopped, as they did following the introduction of epinephrine and nor-epinephrine, but instead remained markedly elevated for the remainder of the 180 minutes they were followed (Fig. 4). These peak levels were 18.9 and 7.4 times higher than the control figures. Also, in the first of these studies (Dog 11), a rise of 1.6 times the control level occurred in the PBI^{131} of the femoral arterial blood. In the other experiment, a small increase in the arterial PBI^{131} of 1.3 times the control activity was noted. A summary of these TSH experiments is found in Table 1.

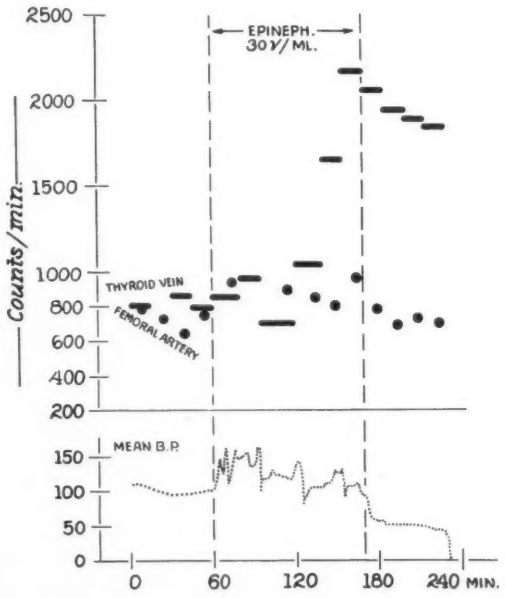

FIG. 5. (Dog 13, hypophysectomized.) The ratio of the peak PBI^{131} level during the infusion period to the control levels was 2.5 in this experiment.

Epinephrine Administration in Hypophysectomized Animals

Infusions of epinephrine in two hypophysectomized dogs produced results comparable to those obtained with epinephrine administered to intact animals (Fig. 5). Latent periods of 35 to 80 minutes were followed by increases of PBI^{131} levels in the thyroid vein which reached peaks 2.5 and 3.2 times as high as the control levels. No significant changes occurred in the PBI^{131} levels of the femoral artery. These findings are summarized in Table 1.

Preliminary Radiopaperchromatographic Studies

Chromatographic separation of the I^{131} labeled compounds in the thyroid vein was carried out during one experiment with epinephrine and one with thyroid stimulating hormone. A comparison of the chromatographic patterns of the PBI^{131} in the thyroid vein before the administration of epinephrine and during the height of the PBI^{131} response to epinephrine is shown in Figure 6. It is apparent that the rise in protein bound radioactivity during the epinephrine infusion is attributable to increases in both I^{131} labeled

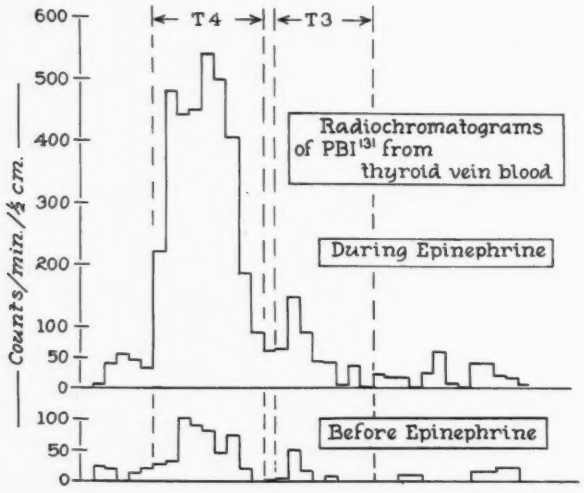

FIG. 6. (Dog 7.) A comparison of the radiopaperchromatograms of equal volumes (3 ml.) of thyroid venous plasma taken before epinephrine and during the height of the response of the thyroid gland to epinephrine. Increases in both radioactive thyroxine (T4) and triiodothyronine (T3) occurred during the infusion of epinephrine.

thyroxine and triiodothyronine. Chromatograms made from femoral arterial blood during the control periods of these experiments and during the infusion of epinephrine showed small peaks of labeled thyroxine with no evidence of triiodothyronine activity. These chromatographic studies of thyroid venous blood following epinephrine and TSH stimulation are at present being continued in additional dogs.

DISCUSSION

Although the effects of epinephrine on thyroid gland activity have been studied in some detail in the past, the results have not been uniform from one species to another and not even within the same species. While it has been found by Soffer and associates (16) that circulating thyroid stimulating hormone is increased in thyroidectomized dogs after epinephrine ad-

min stration, Botkin (21) and Soffer (22) have reported a decrease in the uptake of I^{131} in rats after epinephrine injection. Perry (23) has demonstrated a rise in the basal metabolic rate and hypertrophy of thyroid tissue in rats following epinephrine administration. Goetsch (24), on the other hand, found no histological changes in the thyroid glands of guinea pigs after daily epinephrine injections. He did, however, find changes suggesting increased activity in the thyroid glands of rabbits after a similar course of epinephrine. Money (25) has reported a decrease of the I^{131} uptake in rats after two and three weeks of daily epinephrine injections, but no changes after one week. He noted, however, a significant decrease in the uptake of I^{131} only two hours after a single injection of epinephrine. Reiss and associates (26) have studied the effect of epinephrine on the I^{131} uptake by the thyroid gland in man, and have observed increases which have reached their maximums three to four hours after a single dose of epinephrine. There is evidence that epinephrine may not only influence the formation of thyroid hormones, but that it may influence the peripheral metabolism of these hormones. Thus, several groups of investigators (17, 18, 27) have demonstrated an acute decrease in serum protein bound iodine in thyroidectomized rats following epinephrine administration.

There has been relatively little investigation of the effect of epinephrine on thyroid function in dogs (16), the species with which this report is concerned. The data which have been obtained in this species and others, however, by the use of various indices of thyroid stimulation (size of gland, I^{131} uptake, serum PBI^{131} levels) have often indicated the ability of epinephrine to exert a stimulating effect on the activity of the thyroid gland. The measurement of the radioactive protein bound iodine in thyroid venous blood, however, as described in this study on dogs, provides for the first time a direct and more sensitive method for studying the effect of epinephrine on the actual release of thyroid hormones from the thyroid gland.

The data from the control periods are of interest in that they demonstrate the relative levels of radioactivity of thyroid venous and peripheral arterial blood that one might have predicated on the basis of the known physiologic functions of the thyroid gland. The continuous release of thyroid hormones from the thyroid gland in very small quantities during "resting states" (28, 29) is reflected by the elevated measurements of PBI^{131} in the thyroid vein as compared to the peripheral arterial values of PBI^{131} during the control periods (Figs. 1, 2, 3, 4, 5). The remarkable ability of the thyroid gland to concentrate inorganic iodide some 100 times has been recognized for many years and represents an unusual activity of this gland. This function of the thyroid gland is demonstrated by the fact that the *total* I^{131} activity in the thyroid vein averaged 13% lower than in the simultaneously drawn peripheral arterial samples, in spite of the higher thyroid venous PBI^{131} values, indicating a smaller amount of inorganic radioactive iodide in the thyroid vein than in the peripheral arterial blood.

The results of the seven epinephrine and three nor-epinephrine experiments (Table 1) indicate the ability of these substances to produce prompt and striking increases in the release of thyroid hormones from the thyroid gland of dogs. Additional experiments were then carried out to elucidate the exact site of this action of epinephrine. Two possible pathways of epinephrine stimulation were considered. Indirect stimulation of the thyroid gland via hypothalamic anterior pituitary activation furnishes one possible route. The possibility that epinephrine and nor-epinephrine directly stimulate the thyroid gland to release its hormones was also considered.

Accordingly, experiments with thyroid stimulating hormone and experiments utilizing hypophysectomized dogs were carried out to help elucidate whether the effects of epinephrine and nor-epinephrine on the secretory activity of the thyroid gland were direct or indirect.

The intravenous administration of thyroid stimulating hormone to two dogs was followed by a prompt increase in the release of thyroid hormones from the thyroid gland (Table I, Fig. 4). The data obtained in the TSH experiments were grossly similar to those noted in the experiments with epinephrine and nor-epinephrine, and are compatible with the conclusion that the response of the thyroid gland to epinephrine could be mediated by the anterior pituitary release of thyroid stimulating hormone.

Certain differences, however, were noted between the TSH and epinephrine responses. After the administration of thyrotropin, the PBI^{131} rise in the thyroid vein occurred somewhat more rapidly and persisted at its peak values longer, even after TSH was withdrawn (Fig. 4), than was noted in the experiments with epinephrine and nor-epinephrine. Also, the mean rise in the PBI^{131} compared to the resting values was greater after thyrotropin administration than following the other two substances. These variations, however, may be a reflection of the relative difference in dosage of the administered materials.

Thus, it should be noted that the administration of 10 to 20 units of thyroid stimulating hormone to a 15 kg. dog represented a relatively large dose, whereas the amounts of epinephrine and nor-epinephrine infused were within physiologic limits. The high dosage of TSH was utilized because it was feasible to follow the thyroid venous PBI^{131} values in these dog experiments for a total of only four to six hours, and as prompt a thyrotropin effect as possible was desired. Pertinent to these experiments with TSH are the data of D'Angelo (30), who was unable to detect any rise in blood levels of thyroid stimulating hormone in rats after single and multiple injections of epinephrine. Thus, the data acquired in the two TSH experiments reported do not conclusively corroborate or refute the possibility that epinephrine may stimulate secretion from the thyroid gland via anterior pituitary release of thyrotropin.

On the other hand, the data acquired in hypophysectomized dogs, demonstrating a prompt rise in the thyroid venous PBI^{131} following epi-

nephrine infusion comparable to that seen in the intact dog, indicate that epinephrine does act directly on the thyroid gland. Although these experiments on hypophysectomized dogs do not rule out the possibility that the epinephrine-induced release of thyroid hormones may also arise in part via stimulation of the anterior pituitary-thyroid axis, they do suggest that the major pathway of epinephrine activation of secretion by the thyroid gland is a direct effect of epinephrine on the gland itself. It is also conceivable in view of the variety of metabolic and cardiovascular effects of epinephrine that even in the hypophysectomized animal, the changes in secretion by the thyroid gland following epinephrine may not be primary effects upon the gland itself.

The absence of any rise in PBI^{131} levels in the peripheral arterial samples during epinephrine and nor-epinephrine stimulation warrants further comment in view of the concomitant increases of the PBI^{131} in the thyroid vein. At least two factors may help explain these findings. Undoubtedly, there is a marked dilution effect as the PBI^{131} in the thyroid vein mixes with the entire intravascular compartment. This dilution could completely mask any tendency for the PBI^{131} concentration in the peripheral artery to rise. In addition, an increase in tissue "utilization" or metabolism of thyroid hormone during epinephrine administration has been postulated (23), and such a mechanism may be responsible in part for the failure of the PBI^{131} to rise in the peripheral blood. Indeed, in a few studies (Fig. 3) the PBI^{131} values of the femoral artery decreased slightly as the experiment progressed. This minimal decrease in the PBI^{121} may be attributed to the slight hemodilution that took place during the experiments as serial blood samples were removed and 5 per cent dextrose-in-saline slowly infused. The increase of PBI^{131} levels in the femoral artery that appeared during the administration of thyrotropin may be a function of the quantitatively greater release of PBI^{131} that occurred following the TSH dose employed than after the administration of epinephrine and nor-epinephrine. Thus, any dilution effect and peripheral utilization factor may have been overcome by the relatively large amount of PBI^{131} released following the TSH administration.

The radiopaperchromatographic studies reported are of a preliminary nature and are being expanded at the present time. The chromatograms demonstrated that the rise in PBI^{131} that took place during one epinephrine infusion was attributable to increases in both radioactive thyroxine and triiodothyronine. The rise of triiodothyronine noted in the thyroid venous blood is an important finding, since it furnishes direct evidence that this hormone can be released from the thyroid gland. Triiodothyronine has been identified in the thyroid gland and blood of animals (31) and man (1, 2, 32). It is not clear, however, whether the circulating level of triiodothyronine is primarily the result of thyroid gland secretion or is maintained by the peripheral deiodination of thyroxine. There is experimental

evidence for the latter process in man (33), animals (34, 35) and in *in vitro* systems (36). Previous direct evidence to support the secretion of triiodothyronine by the thyroid gland has been furnished by Taurog *et al.* (37), who described the presence of triiodothyronine in the thyroid vein of seven sheep and one horse. It is possible that both mechanisms may be responsible in part for the circulating level of triiodothyronine.

Preliminary data would indicate, therefore, that rapidly acting, potent triiodothyronine may be secreted by the thyroid gland of the dog in response to the acutely stressful situation associated with epinephrine elaboration, and thus may serve as an "emergency" type of hormone. Additional chromatographic studies of thyroid venous blood following epinephrine administration are currently in progress. Increased thyroxine secretion demonstrated following the epinephrine infusion might also increase the peripheral production of triiodothyronine by means of the tissue deiodination mechanism previously mentioned.

Acknowledgment

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PURIFICATION OF GONADOTROPHINS DERIVED FROM URINE AND PITUITARY GLANDS OF HUMAN BEINGS: OBSERVATIONS ON THEIR ELECTROPHORETIC BEHAVIOR AND BIOLOGICAL ACTIVITY[1]

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ABSTRACT

A method for purifying human urinary gonadotrophins which is also applicable to purification of human pituitary gonadotrophins is described. Ninety-five to ninety-seven percent of the inactive impurities and toxic substances were eliminated without detectable loss of gonadotrophic activity.

Bioassay of the purified products in immature hypophysectomized male and female rats indicated the presence of the three gonadotrophic activities.

Electrophoretic analysis at pH 8.6 of both the urinary and pituitary preparations revealed three components migrating as anions. Electrophoretic fractionation and bioassay of the fractions on immature hypophysectomized male and female rats failed to demonstrate that each of the three gonadotrophic activities (FSH, ICSH, and Luteotrophin) was associated with a different electrophoretic component, and indicated that all three of them may be associated with the central component.

This evidence suggests that the human urinary and pituitary gonadotrophins are similar to each other and that either they are high molecular weight protein-like substances or that they are associated with such substances.

METHODS for concentrating urinary gonadotrophins currently in use, such as alcohol precipitation (1), kaolin absorption (2), and ultrafiltration (3, 4), yield products containing many other proteins and nonprotein substances. This has limited the characterization of human urinary gonadotrophins, as the toxicity of these extracts to hypophysectomized rats hampers biologic assay and the extraneous substances interfere with definitive analysis. The present report deals with a method that concentrates the usual ultrafiltration end product 20 to 30 times without significant loss of gonadotrophic activity. This permits electrophoretic analysis and biologic assay in hypophysectomized rats without causing toxic effects.

MATERIALS AND METHODS

Urine from normal adult male and female human subjects or from patients with pre-

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pulseral seminiferous tubule failure (Klinefelter's Syndrome) was collected as voided and preserved at 1° C. as each 24-hour specimen accumulated from each subject, it was concentrated by ultrafiltration in the usual manner to recover the gonadotrophins. The dry residue was stored at 1° C. Later, for bioassay and for subsequent purification, the dry residue was extracted with distilled water. Each 24-hour aliquot was extracted three times using 2 cc. of distilled water per extraction. The clear extracts obtained by centrifugation were pooled and designated as the "Initial Extract."

Human pituitaries were obtained immediately after traumatic death from presumably normal adult men and women, dried with acetone, and stored at 1° C.[3] Just prior to extraction the pituitaries were resoaked in acetone and the anterior lobes separated off redried, pulverized, and weighed as dry powder. The powder was extracted three times with isotonic saline (20 cc. per gm.) at pH 9.0 and the pooled extracts constituted the "Initial Extract" which was then subjected to the purification procedure.

Extracts to be assayed in intact animals were always made up to 6 cc. with water so that 1 cc. was injected subcutaneously, twice daily, in each 24-day-old *intact* female Sprague-Dawley rat for 3 days. Twenty-four hours following the last injection the animals were sacrificed by rapid decapitation and the weights of the ovaries and uteri were recorded.

Electrophoretic analyses were performed with a Klett apparatus utilizing the 11 cc. electrophoretic cell and veronal buffer of pH 8.6, ionic strength 0.1.

The migrating proteins in the electrophoretic cell were sampled with a sampling device previously described (5) and the fractions thus obtained were assayed in immature hypophysectomized male and female Sprague-Dawley rats (6). Hypophysectomy was performed when the rats were 22 days of age[4] and the assays started when 24 days old. The fractions to be assayed were diluted with 5% dextrose so that each rat received a total dose of 5 cc. (0.5 cc. injected subcutaneoulsy twice daily for 5 days). Forty-eight hours following the last injection the animals were sacrificed by rapid decapitation and the weights of the ovaries, uteri, seminal vesicles, anterior prostates, and testes were recorded.

PURIFICATION PROCEDURE

Human Urinary Gonadotrophins:

Albumins, globulins, acid insoluble mucoproteins and glycoproteins have been demonstrated in the urine of normal human subjects (7). The acid insoluble mucoproteins are precipitable by acidification to pH 3.8, while the globular proteins are removed following denaturation by shaking with water immiscible organic solvents such as chloroform or ethyl ether. The remaining proteins, which contain the gonadotrophic activity, can be further fractionated by ethanol precipitation or by salting out with ammonium sulfate. These principles were utilized in the numerous preliminary procedures which led to the development of the method described here. The objective of the proposed method was to obtain a purified non-toxic material without loss of gonadotrophic activity. We have, therefore, included in the purification scheme, as finally evolved, only those steps which yielded inactive waste products. All procedures were performed in a cold room at 1° C.

The "Initial Extract" of each 24-hour urine aliquot from the same patient was pooled for convenience of manipulation and the pH (usually between 6.5 and 7.0) adjusted to 3.8 by the slow addition of 1.0N HCl with constant stirring. The suspension was centrifuged and the clear aqueous phase recovered.

[3] We wish to thank Dr. Kenneth Wade Thompson, Orange, New Jersey, for supplying the human pituitaries used in these studies.

[4] Hypophysectomies performed by Hormone Assay Laboratoties, Chicago, Illinois.

SCHEME 1

DRY POWDER OBTAINED BY ULTRAFILTRATION OF URINE OR ACETONE-DRIED PITUITARY GLANDS

	↓
Residue. Discard.	"Initial Extract." Adjust pH to 3.8; centrifuge.
	↓
Precipitate. Discard.	Supernatant solution. Shake with equal volume of ethyl ether; centrifuge.
	↓
Ether phase plus Precipitate. Discard.	Aqueous phase. Add 0.1 volume of saturated NaCl; adjust pH to 7.0; add ethanol to 50%; centrifuge.
	↓
Precipitate. Discard.	Supernatant solution. Add more ethanol to 85%; centrifuge.
	↓
Supernatant fluid. Discard.	"Product 1." Dry; dissolve in 5 cc. water per 25 cc. of "Initial Extract"; add 0.1 volume of saturated NaCl; adjust pH to 6.0; add ethanol to 55%; centrifuge.
	↓
Precipitate. Discard.	Supernatant solution. Add 0.15 volume of saturated NaCl; ethanol to 75%; centrifuge.
	↓
Supernatant fluid. Discard.	"Product 2." Dry; dissolve in 2 cc. water per 25 cc. of "Initial Extract"; add 0.1 volume of saturated NaCl; adjust pH to 4.8; add ethanol to 50%; centrifuge.
	↓
Precipitate. Discard.	Supernatant solution. Add 0.1 volume of saturated NaCl; ethanol to 75%; centrifuge.
	↓
Supernatant. Discard.	"Product 3." Dry; dissolve in 4 cc. water per 25 cc. of "Initial Extract"; add ammonium sulfate to 0.4 saturation; centrifuge.
	↓
Precipitate. Discard.	Supernatant solution. Add ammonium sulfate to 0.6 saturation; centrifuge.
	↓
Supernatant fluid. Discard.	Precipitate. Dissolve in water; dialyze; lyophilize.
	↓
	"Final Product"

WORKING FLOW SHEET SUMMARIZING THE PURIFICATION OF THE HUMAN URINARY AND PITUITARY GONADOTROPHINS

The ionic strength of the aqueous phase was then increased by the addition of 0.1 volume of saturated sodium chloride and the pH adjusted to 7.0 with 1.0N NaOH. Cold 95% ethanol was added to obtain a final concentration of 50% by volume, and the suspension centrifuged until clear. The gonadotrophins were precipitated from the supernatant solution by raising the ethanol concentration to 85% by volume. After one hour the precipitate was centrifuged, washed with absolute ethanol, ethyl ether, and dried in a stream of air. This was designated as "Product 1."

The dry "Product 1" was subsequently dissolved in cold distilled water using 5 cc. for each 25 cc. of the "Initial Extract." One tenth volume of saturated solution of sodium chloride was added, and the pH adjusted to 6.0 with 1.0N HCl. Cold 95% ethanol was then added to a final concentration of 55% by volume. Complete precipitation occurred after a few hours in the cold and the suspension was then centrifuged. The ionic strength of the clear supernatant was increased by adding a saturated solution of sodium chloride, 1.5 times the volume previously added, and the gonadotrophins were precipitated by raising the ethanol concentration to 75%. The suspension was centrifuged until clear and the precipitate washed and dried as was the previous one. This was designated as "Product 2."

"Product 2" was dissolved in cold distilled water using 2 cc. for each 25 cc. of the "Initial Extract." The ionic strength was increased by the addition of 0.1 volume of

saturated solution of sodium chloride. The pH was adjusted to 4.8 with 1.0N HCl and cold ethanol was added to a final concentration of 50% by volume. Precipitation was complete after a few hours in the cold, and the suspension was centrifuged until clear. A volume of saturated solution of sodium chloride, equal to that which was added previously, was then added to the supernatant solution and the gonadotrophins were precipitated by increasing the ethanol concentration to 75% by volume. The suspension was centrifuged until clear, and the precipitate washed and dried as previously. This was designated as "Product 3."

"Product 3" was dissolved in cold distilled water using 4 cc. for each 25 cc. of the "Initial Extract." Enough crystalline ammonium sulfate was added to give a concentration of 0.4 saturation (28.2%). The suspension was centrifuged until clear, and the gonadotrophins were then precipitated from the supernatant solution by the addition of crystalline ammonium sulfate to raise the concentration to 0.6 saturation (42.4%). The precipitated gonadotrophins were recovered by centrifugation, dissolved in a small volume of distilled water, dialyzed in a cellophane tube[5] against cold running water until free from sulfate, and lyophilized. The lyophilized product, a white amorphous material very soluble in water, was designated as the "Final Product." Scheme 1 is a convenient flow sheet summarizing this method.

RESULTS

Elimination of Impurities: This method eliminates 95% to 97% of the inert impurities found in the residue of ultrafiltered urine. This is shown in Tables 1 and 2. In Table 1 the dry material in 24 hours of the "Initial Extract" weighed 107.3 mg. but after purification a 24 hour aliquot of the final product weighed only 5.2 mg. The toxic substances are eliminated to such an extent that doses up to 100 hours of urinary output can be injected into a single immature hypophysectomized rat without ill effects. Furthermore, partially purified products which were obtained by a simplification of this method have been injected into humans without toxic effects (8).

Similar results were obtained when the human pituitary powder was processed in the same manner. Approximately 98% of the inert material was removed.

Recovery of the Gonadotrophins: The elimination of the contaminating substances resulted in no detectable loss of the gonadotrophic activity as can be seen from Table 1, where the products obtained during the purification of a urine were assayed in doses representing equal hours of urinary output. The recovery of gonadotrophins was complete whether urine of high potency (Table 2, Urine I and II) or low potency (Table 2, Urine III) was used. The gonadotrophins were thus concentrated by this method 20 to 33 times over their concentration in the "Initial Extract." There may in fact, be some enhancement in the response of the rat ovary to the more purified materials (in Table 1 compare the response to "Initial Extract" with the response to "Final Product").

[5] Cellophane sausage casing, size 27/32, obtainable from Visking Corporation, Chicago, Illinois, was used.

TABLE 1. ASSAY OF THE GONADOTROPHIC ACTIVITY OF A URINE THROUGHOUT ITS PURIFICATION IN *Immature Intact* FEMALE RATS

Material injected	Total dose per rat*		Uterine weight, drained, mg.	Ovarian weight, mg.
	Hours of original urine	Mg. of dry powder		
Initial Extract	24	107.3	129	53.8
	24	107.3	130	93.7
	24	107.3	129	67.3
	24	107.3	80	125.8
		Mean Weight	117	85.2
Product 1	24	27.3	82	90.7
	24	27.3	84	79.0
	24	27.3	102	91.3
	24	27.3	115	96.0
		Mean Weight	96	89.8
Product 2	24	17.3	94	88.5
	24	17.3	94	139.8
	24	17.3	122	125.0
	24	17.3	112	89.5
		Mean Weight	106	110.7
Product 3	24	13.7	125	99.5
	24	13.7	84	114.3
	24	13.7	99	114.1
	24	13.7	132	80.0
		Mean Weight	110	102.0
Final Product	24	5.2	98	101.5
	24	5.2	110	107.6
	24	5.2	102	95.3
	24	5.2	125	116.2
		Mean Weight	109	105.2
Uninjected Controls			42	15.0
			17	13.2
		Mean Weight	30	14.1

* Each total dose was diluted to 6 cc. so that each assay rat received 1 cc. subcutaneously twice daily for 3 days.

In Table 2 it can be seen that purifying the pituitary powder resulted in no loss of gonadotrophic potency. Each assay rat received a total dose representing the material obtained from 30 mg. of dry pituitary powder.

Electrophoretic Analysis: One hundred (100) mg. of the "Final Product" of Urine I representing 500 aliquot hours; 100 mg. of dialyzed and lyophilized "Initial Extract" of human pituitary glands representing 0.5 gm. of dry pituitary powder; and 20 mg. of pituitary "Final Product" representing 4.3 gm. of dry pituitary powder, were each dissolved in 11 cc. of veronal buffer and separately analyzed under identical experimental conditions.

TABLE 2. COMPARISON OF THE GONADOTROPHIC ACTIVITY OF THE INITIAL EXTRACTS AND THE FINAL PRODUCTS OF THREE URINE SPECIMENS AND HUMAN PITUITARIES INJECTED INTO *immature intact* FEMALE RATS

Material injected	Source of gonadotrophin	Number of rats	Total dose per rat*		Mean weight of uterus, drained, mg.	Mean ovarian weight, mg.
			Hours of original urine	mg.		
Initial Extract	Urine I	2	5	24.4	101	97.3
	Urine II	2	12	54.9	133	59.9
	Urine III	3	24	108.0	102	14.1
	Human Pituitaries	3	(30 mg.†)	8.2	114	217.4
Final Product	Urine I	2	5	1.0	92	103.1
	Urine II	2	12	2.7	186	84.0
	Urine III	3	24	3.5	98	14.6
	Human Pituitaries	3	(30 mg.†)	0.14	104	245.5
Uninjected Controls	—	4	—	—	36	14.2

* Each total dose was diluted to 6 cc. so that each assay rat received 1 cc. subcutaneously twice daily for 3 days.

† Each assay rat received extract representing 30 mg. of acetone dried anterior pituitary powder.

Figure 1 depicts the descending boundaries obtained from these analyses. Three major components *a*, *b*, and *c*, common in both the urinary and pituitary purified gonadotrophins, are observed moving away from the false boundary ϵ as anions. The electrophoretic mobilities at pH 8.6 and the relative concentrations of these components have been calculated and tabulated in Table 3. The absolute concentrations of *a*, *b*, and *c* have also been calculated, assuming that no material is present under the false bound-

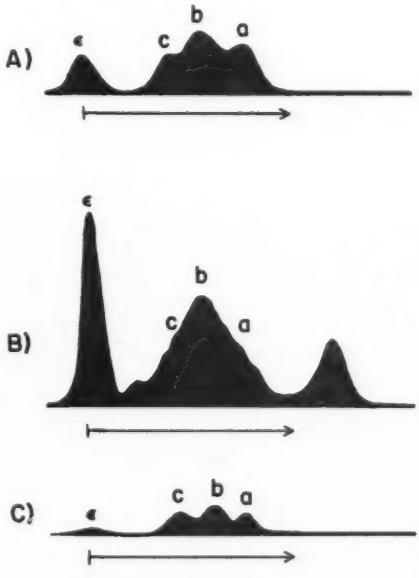

FIG. 1. Descending electrophoretic patterns of: A) purified human urinary gonadotrophins (Urine I) from a subject having puberal seminiferous tubular failure; B) initial extract of human pituitaries; C) purified human pituitary gonadotrophins. Patterns were recorded after 90 minutes migration in veronal buffer pH 8.6, ionic strength 0.1, under the influence of 7.4 volts/cm.

TABLE 3. ELECTROPHORETIC MOBILITIES AND CONCENTRATIONS OF COMPONENTS *a*, *b*, AND *c* OF THE FINAL PRODUCT

Component	Electrophoretic mobility, cm.^{2} sec.$^{-1}$volt^{-1} $\times 10^5$	Concentration			
		Urine I		Human pituitaries	
		% of total	Gm./100cc.	% of total	Gm./100cc.
a	−5.2	28	0.27	26	0.05
b	−3.8	54	0.50	42	0.08
c	−2.6	18	0.15	32	0.06

aries. The "Initial Extract" of the human pituitaries shows the presence of a number of components migrating in the region of *a*, *b*, and *c*, and in addition one slower and one faster component.

Electrophoretic Fractionation and Biological Activity: The electrophoresis of the "Final Product" of Urine I was continued until the component *a* had migrated the full length of the cell. Several fractions were withdrawn with the aid of the sampling device. No such fractions could be obtained from the "Final Product" of the pituitary glands due to difficulties in detecting the extremely weak boundaries after their full migration. Figure 2 shows the appearance of the ascending and descending boundaries of the urinary "Final Product" placed along the sides of a diagrammatic sketch of the electrophoretic cell, which illustrates the degree of separation of the components *a*, *b*, and *c*, at the time of fractionation. Three fractions were withdrawn from each limb, indicated by the horizontal lines in Figure 2, and marked with Roman numerals followed by the letters "A" or "D." The components present in each fraction are illustrated in Figure 2, and are tabulated in Table 4.

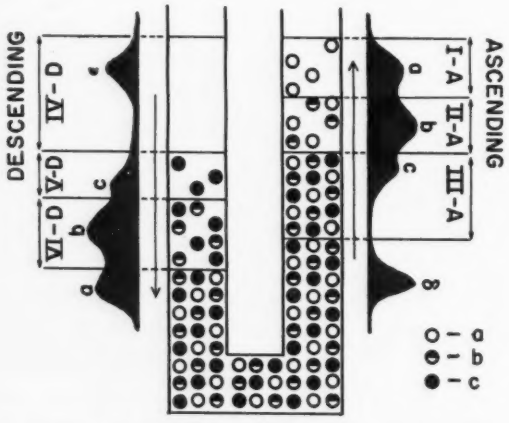

FIG. 2. Electrophoretic patterns of purified urinary gonadotrophins and diagramatic sketch of the electrophoretic cell illustrating the degree of the separation of the components *a*, *b*, and *c* at the time of the fractionation.

TABLE 4. ASSAY OF THE ELECTROPHORETIC FRACTIONS OF THE PURIFIED URINARY GONADOTROPHINS (URINE I) ON *immature hypophysectomized* RATS

Fraction	Total Dose per animal*				Female immature hypophysectomized rats				Male immature hypophysectomized rats			
	Dry weight in mg.				Uterine with fluid	Weight mg. without fluid	Ovarian weight, mg.	Ovarian appearance	Seminal vesicles, mg.	Anterior prostate, mg.	Testes, mg.	Epididymis, mg.
	Total	*a*	*b*	*c*								
I-A	0.3	0.3	traces	0	—	16	5.5	Infantile	5.1	5.2	125	25.9
	0.3	0.3	traces	0	—	16	4.9	Infantile	7.0	6.7	125	26.6
	0.3	0.3	traces	0	—	19	5.7	Infantile	5.0	5.7	131	27.4
II-A	1.3	0.7	0.6	traces	391	145	57.7	Clear foll.	10.4	22.1	325	51.3
	1.3	0.7	0.6	traces	435	131	73.6	Clear foll.	14.5	24.2	388	62.5
III-A	2.3	0.7	1.3	0.3	334	137	82.4	Clear foll.	20.6	27.3	398	63.9
	2.3	0.7	1.3	0.3	471	201	89.3	Clear foll.	17.3	52.4	378	—
	2.3	0.7	1.3	0.3	—	123	100.7	Clear & semi-opaque foll.	16.9	28.3	351	65.7
IV-D	traces	0	0	traces	—	18	4.2	Infantile	4.5	5.4	120	27.9
	traces	0	0	traces	—	19	3.3	Infantile	6.5	5.9	137	34.5
	traces	0	0	traces	—	—	—	—	6.6	6.6	140	27.4
V-D	0.2	0	traces	0.2	—	15	7.4	Infantile	7.8	13.3	300	44.8
	0.2	0	traces	0.2	89	62	6.9	Clear foll.	8.3	17.2	288	42.4
VI-D	1.1	traces	0.7	0.4	178	122	74.1	Lge. clear foll.	15.1	19.2	337	46.3
	1.1	traces	0.7	0.4	132	119	65.8	Lge. clear foll.	11.8	26.3	331	46.7
	1.1	traces	0.7	0.4	162	91	37.8	Clear foll.	—	—	—	—
Final Product (Unfractionated)	2.4	0.7	1.3	0.4	125	107	89.7	Clear semi-opaque foll.	30.7	47.3	435	64.9
	2.4	0.7	1.3	0.4	146	134	125.1	Clear semi-opaque foll.	20.4	29.3	386	50.1
	2.4	0.7	1.3	0.4	179	176	103.2	Clear semi-opaque foll.	—	—	—	—
Uninjected Controls					—	18	5.4	Infantile	7.6	7.0	142	22.4
					—	15	4.7	Infantile	6.0	6.3	118	22.4
					—	15	3.9	Infantile	7.1	6.1	132	28.0
					—	18	5.7	Infantile	7.5	6.8	134	27.5
					—	—	—	—	6.2	7.6	145	23.8

* The total dose was diluted to 5 cc. so that each assay rat received 0.5 cc. subcutaneously twice daily for 5 days.

The electrophoretic fractions obtained were dialyzed against water until free from buffer salts before they were assayed in both female and male immature *hypophysectomized* rats. A total dose of 0.26 cc., representing the purified material derived from 12 hours of the original urine, was diluted up to 5 cc. and administered to each rat. The unfractionated "Final Product" was assayed concurrently. No direct determination of the nitrogen or dry weight present in each fraction was undertaken because of the limited amount of the available material. However, the total dry weight as well as that of each of the components present in the total dose given to each animal were estimated from the electrophoretic patterns, assuming that the mean concentration of a component in the region occupied by its boundary is one half its concentration in the analyzed solution. These estimates and the results of the assays are recorded in Table 4. Because of diffusion and possible convection during the electrophoresis and subsequent fractionation, it is assumed that each fraction is contaminated with "traces" of the adjacent component.

The progestational uterus devoid of fluid obtained by the injection of Fraction III-A, and the progestational uterus nearly devoid of fluid obtained with the unfractionated "Final Product" indicate that all three gonadotrophic effects are present in the purified human urinary gonadotrophins (6). Fractions IV-D (containing traces of component *c*), and I-A (*a*, plus traces of *b*) did not cause stimulation, thus indicating the absence of FSH and ICSH from the component *a* and the false boundary ϵ. Minimal stimulation was observed in the animals which received Fraction V-D (*c*, plus traces of *b*), but significant responses were obtained from the assay of the rest of the fractions, all of which are rich in component *b*.

DISCUSSION

Although the three gonadotrophins, FSH, ICSH, and Luteotrophin, when prepared from animal pituitary glands, appear to be proteins distinct from each other (9), it is not known whether the human urinary and pituitary gonadotrophins are also substances distinct from each other, or only one substance possessing all three gonadotrophic effects. Inconclusive results were obtained from previous attempts to separate them by chemical fractionation (10), and to characterize them by paper electrophoresis (11, 12). The utilization of free boundary electrophoresis in the present work has definitely demonstrated that the human gonadotrophins move as anions at pH 8.6, and has revealed the existence of three major components in the purified products. The fact that no loss of any of the three gonadotrophic activities was observed after extensive dialysis of human urinary or pituitary preparations, and their apparent migration as anions on electrophoresis at pH 8.6, indicate that they are protein-like substances of high molecular weight, or that they are strongly associated with such sub-

stances. The feasibility of purifying human gonadotrophins either from urine or pituitary glands by subjecting them to identical protein fractionation procedures suggests that, regardless of the source, they are similar to each other.

It is tempting to believe that each of the observed electrophoretic components (*a*, *b*, and *c*) represents a different gonadotrophin, one being FSH, another the ICSH, and the third the Luteotrophin. The fact that these components were the only ones observed in both the urinary and the pituitary "Final Products" adds impetus to this belief.

Thus, the observation that the component *a* alone (Fraction I-A) does not cause any stimulation in the hypophysectomized male or female rat, but in the presence of *b* and *c* (Fraction II-A, and "Final Product") produces progestational uteri, could be interpreted as meaning that component *a* is the Luteotrophin. Component *c*, which when present alone (Fraction V-D) causes some stimulation in the male, could be interpreted as being the ICSH, and component *b*, which whenever present in the fractions (along with some *c*) causes a good stimulation, could be thought of as being the FSH. The minimum stimulation caused by Fraction V-D (component *c* or ICSH) in one of the female rats, could be explained by the unavoidable contamination with the adjacent component *b* (FSH).

However, assuming that the weight response of the anterior prostate and the seminal vesicles is primarily a measure of ICSH activity, and that the increase of uterine and ovarian weight indicates the total gonadotrophic activity, we can compare these activities to the concentration of each of the components *a*, *b*, *and c* present in each fraction. Inspection of Table 4 shows that both the ICSH and the total gonadotrophic activities increase as the concentration of component *b* increases, and that there is no correlation of either one of them with the concentration of *a* or *c*. This suggests that both the FSH and the ICSH activities are associated with component *b*.

Thus, despite the finding of three components migrating separately in the electrophoretic cell when highly purified human urinary and pituitary gonadotrophins were subjected to analysis, biological assay failed to confirm that these three components represent FSH, ICSH and Luteotrophin. Since separation of the three gonadotrophin factors was not demonstrated in these experiments, we must continue to consider the possibility that in the human being a single substance having three actions may exist.

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THE EFFECT OF HYPOGLYCEMIA ON THE ADRENAL SECRETION OF EPINEPHRINE AND NOREPINEPHRINE IN THE DOG[1]

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ABSTRACT

A modification of the sensitive fluorometric procedure of Weil-Malherbe and Bone has been applied to a study of the changes in epinephrine and norepinephrine in adrenal venous and peripheral venous plasma of 24 lightly anesthetized mongrel dogs.

Insulin hypoglycemia produced a marked increase in the adrenal secretion of epinephrine and a much less marked increase of norepinephrine. The infusion of glucose produced a rapid decline in the elevated secretion rate of both epinephrine and norepinephrine while the administration of amphenone produced no significant change. An increase in both epinephrine and norepinephrine in response to hypoglycemia was observed in peripheral plasma.

THE increased secretion of sympathomimetic amines occurring in response to insulin-induced hypoglycemia was first demonstrated in 1924 by Cannon, MacIver and Bliss (1) and in the same year by Houssay, Molinelli and Lewis (2). In a series of studies extending over several years Satake (3) found an increase in the content of these substances in blood collected directly from the adrenal vein of the hypoglycemic dog. Following the demonstration of the presence of norepinephrine in the adrenal medulla in 1947 by Holtz, Credner and Kroneberg (4) and in adrenal venous blood by Bülbring and Burn in 1949 (5), a small increase of this amine was observed in the adrenal venous blood of the hypoglycemic cat (6). An increase in the epinephrine concentration of peripheral plasma of the hypoglycemic dog has also been reported (7, 8). The observations of total sympathomimetic amine content of adrenal venous blood following hypoglycemia have been evaluated to date exclusively by bioassay techniques. Results of the present study, facilitated by a recently developed sensitive fluorometric procedure to detect changes of epinephrine, norepinephrine and total catecholamines in plasma, indicate that hypoglycemia leads to an increase

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in adrenal medullary secretion of both epinephrine and norepinephrine in the dog, an increase overcome by the administration of glucose but not amphenone.

This work has previously been reported in abstract form (9).

METHODS

The following studies were performed on male mongrel dogs weighing between 15 and 30 kg. They were anesthetized with 30 mg. of pentobarbitol per kg. of body weight and were maintained in the first plane of surgical anesthesia for experiments performed on the same day. The animals used for study 24 hours postoperatively were allowed to recover and were re-anesthetized 30 minutes before the experiment. The right lumbo-adrenal vein was cannulated and a polyethylene choker was placed around the vein proximal to the gland in such a fashion as to make possible the intermittent collection of adrenal venous effluent. Peripheral blood was obtained by an indwelling catheter in the femoral vein. Samples of blood were collected into heparin, chilled in ice and the plasma was separated by centrifugation, frozen, and stored at −4° C. Experiments were begun at intervals from 30 minutes to 24 hours following completion of the operative procedure. The small volumes of adrenal venous blood collected were not restored but peripheral venous samples when taken were replaced by an equivalent volume of donor dog blood. Epinephrine and norepinephrine were determined by a modification of the fluorometric procedure of Weil-Malherbe and Bone (10). In this procedure epinephrine interferes with the determination of norepinephrine when norepinephrine is present at less than 5% of the concentration of epinephrine (11). The resultant values are somewhat higher than would be found if there were no interference. They are noted in the tables with an asterisk (*). Blood glucose was determined by the Nelson modification of the Somogyi procedure (12) and blood 17-hydroxycorticosteroids were determined by the method of Nelson and Samuels (13). Amphenone does not interfere with the measurement of catecholamines or 17-hydroxycorticosteroids in plasma by these methods, but it does contribute very slightly to the reducing substances measured by the glucose procedure.

RESULTS

Studies were performed on 24 animals grouped as follows: 7 animals received insulin; 4 animals received insulin followed by glucose; 5 animals received insulin followed by amphenone and the remaining animals were treated as specified below. The results of the epinephrine and norepinephrine determinations on adrenal vein samples obtained 10 minutes to 24 hours following completion of the surgical procedure are presented in Table 1. No significant difference in the secretion of these substances was found in the samples collected 1 hour, 2–4 hours and 24 hours following

TABLE 1. POSTOPERATIVE EPINEPHRINE AND NOREPINEPHRINE IN ADRENAL VENOUS PLASMA OF 24 ANESTHETIZED DOGS

Time postoperative	No. of samples	No. of animals	Epinephrine, mμg./kg./min. ±S.D.	Norepinephrine, mμg./kg./min. ±S.D.
0–30 min.	6	3	4. 4±4. 9	1. 4±0.87
1 hr.	6	3	0.57±0.10	1. 0±0.84
2–4 hr.	19	9	0.57±0.52	0.45±0.20
22–24 hr.	17	9	0.38±0.47	0.84±0.92

TABLE 2. EFFECT OF INSULIN HYPOGLYCEMIA ON EPINEPHRINE AND NOREPINEPHRINE IN DOG ADRENAL VENOUS PLASMA

Exp. No.	Wt. in kg.	Hours post-operative	Adrenal venous blood levels													
			Substance measured[1]	Control periods		Experimental periods in minutes following injection of insulin[2]										
				1	2	15–24	25–39	40–45	55–69	70–84	85–99	100–114	115–129	120–144	145–159	160–175
1	17.8	3	E	3.0	0.4	2.2		30	54	62		106	101			
			NE	1.9	0.7	0.6		2.9	4.3	4.0		1.6*	4.5*			
			G		72			30		40		43	27			
2†	17.8	24	E	1.3	0.8	2.8		23	27	66						
			NE	0.2	0.0	0.2		2.8	3.8	6.4						
			G			74		38	20	32						
3	19.4	3	E	0.3	0.1	1.8		21	98	45		56	54			
			NE	1.7	1.1	1.9		3.2	4.1*	4.5		6.8	7.4			
			G		73			34	32	27		29	30			
4	16.0	3	E	0.0	0.0	0.0		0.0	1.0	4.1		15	17	11	9.8	4.7
			NE	0.3	0.2	0.1		0.3	0.2	1.6		1.6	2.6	2.3	1.1	1.4
			G		87	61		34	34	23		22	26	32	36	46
5	22.3	3	E	0.8	0.2	0.6		11	17	30		19	25	22	17	7.4
			NE	0.3	0.2	0.1		1.3	1.4	3.0		3.2	3.0	1.3	0.3	1.6
			G		87	54			27			31				64
6	17.1	24	E	0.5	0.7	0.4	4.3	6.4	11	35						
			NE	0.0	0.0	0.0	0.0	0.02	0.6	2.2						
7	23.0	4	E	0.1	0.3	1.7	32	53	76	195	20					
			NE	0.0	0.1	0.2	3.4	0.0	3.8	4.0*	1.5					
8	15.8	4	E	2.2	3.0	6.6	43	47	85		47					
			NE	0.7	0.4	0.0	0	0	0		0					

[1] E = Epinephrine mμg./kg./min. in plasma.
NE = Norepinephrine mμg./kg./min. in plasma.
G = Glucose mg.% in blood.
[2] Administered intravenously in dose of 1 unit/kg.
* Inaccurate estimation of NE (see text).
† Same animal used in experiment 1 and 2.

surgery. The secretion of epinephrine appeared to be elevated in the few samples obtained during the first 30 minutes.

One unit of crystalline zinc insulin per kg. was administered intravenously in a single rapid injection to 7 dogs following the collection of control specimens. The results of epinephrine, norepinephrine and blood glucose determinations on samples of adrenal venous blood collected at intervals for the next 3 hours are shown in Table 2. The mean values are illustrated graphically in Figure 1. Although there was great variation in the magni-

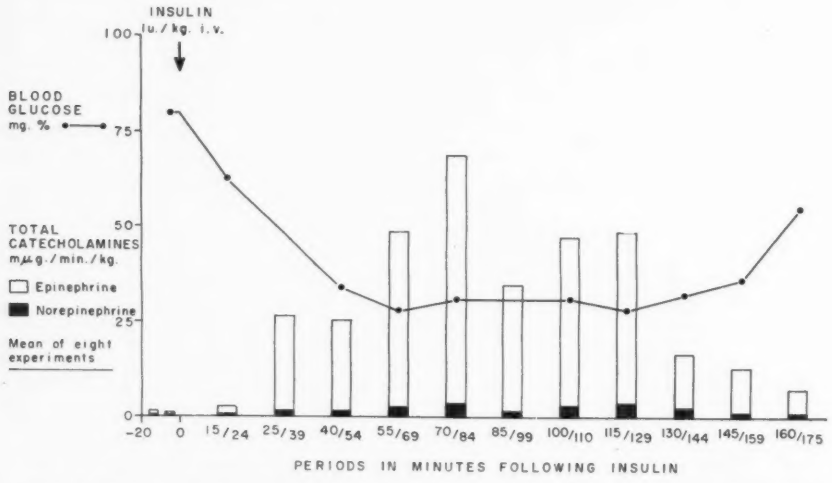

FIG. 1. The effect of insulin induced hypoglycemia on the secretion of epinephrine and norepinephrine from the adrenal. The mean values obtained in the 8 experiments in Table 2 are illustrated.

tude of the response, a marked increase in epinephrine output over control values occurred in each animal. The peak output as measured occurred 60 to 120 minutes after the injection of insulin and was 16 to several hundred fold greater than the control level. It appeared to correspond in an inverse manner to the changes observed in blood glucose concentrations. An increase in the output of norepinephrine was also observed but was much less than that of epinephrine.

The results of a similar study in 4 animals to whom 10–25 gm. of glucose was administered intravenously 70–100 minutes following the administration of insulin are shown in Table 3. Samples obtained at intervals of 5, 10 and 20 minutes following the administration of glucose showed a marked and more rapid reduction in the secretion rate of epinephrine and norepinephrine than was noted in the group not treated with glucose. The mean values obtained in this experiment are illustrated in Figure 2.

These results contrast sharply with an experiment in which the same

TABLE 3. THE EFFECT OF GLUCOSE ON EPINEPHRINE AND NOREPINEPHRINE IN DOG ADRENAL VENOUS PLASMA FOLLOWING INSULIN ADMINISTRATION

Exp. No.	Wt. in kg.	Hours post-operative	Substance measured[1]	Adrenal venous blood levels										
				Control periods		Experimental periods in minutes following injection of insulin[2]						Minutes following injection of glucose[3]		
				20–11	10–1	15–24	25–39	40–54	55–69	70–84	85–99	5	10	20
9	18	½	E	6.1	6.2	13	24	41	49	57	38	13	13	
			NE	1.9	0.9	2.5	3.8	6.0	6.0	5.0	3.7	2.0	2.0	
			G		106	92	49		46		40			
10	17	4	E	2.5	0.4		1.4	4.6	106	50	63		7.4	
			NE	6.0	2.1		5.0	4.0	10	3.0	9.0		2.0	
			G		62				25		28			
11	24	24	E	0.0	1.0	6.1	30	32	69				0.8	7.7
			NE	0.0	0.0	0.3	2.4	2.1	7.1				0.0	0.2
			G		108		71							
12	22	4	E	0.3	0.3	0.4		13		52	76		5.3	
			NE	0.2	0.3	0.4		1.2		4.5	4.0		1.3	
			G		81	50		38		25	19		264	

[1] E = epinephrine mμg./kg./min. in plasma.
NE = norepinephrine mμg./kg./min. in plasma.
G = glucose mg. % in blood.
[2] Administered intravenously in dose of 1 unit/kg.
[3] Administered intravenously in dose of 10–25 grams at 100 min. in experiments 1, 2, and 4 and at 70 min. in experiment 3.

dose of insulin was administered intravenously simultaneously with 30 gm. of glucose to a 30 kg. dog. An increase in the adrenal secretion of norepinephrine and epinephrine failed to occur in this animal.

The administration of insulin at a dosage of 2 units per kg. to two animals as a single injection induced changes similar in magnitude to those

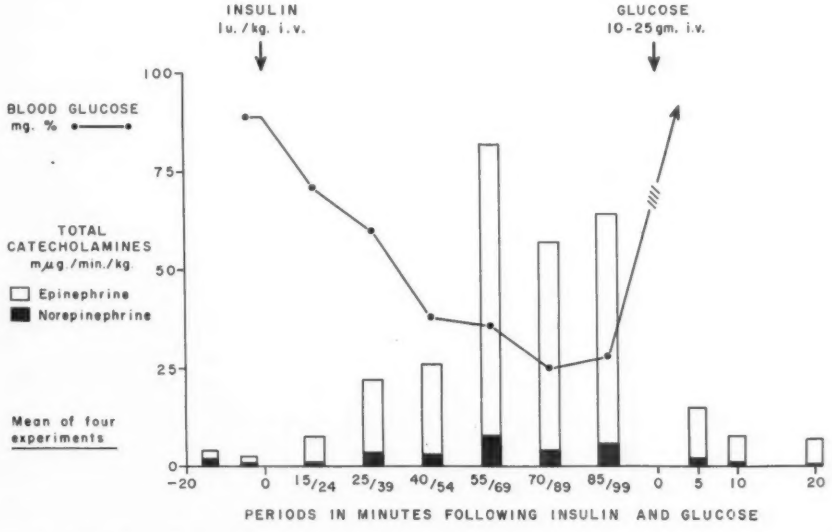

FIG. 2. The effect of glucose administration on the secretion of epinephrine and norepinephrine by the adrenal during hypoglycemia. The mean values obtained in the 4 experiments in Table 3 are illustrated.

observed after the injection of 1 unit. The intravenous infusion of one unit of insulin per kg. in normal saline over 90 minutes resulted only in a slightly delayed peak in a third animal. These three experiments are not included in the tables.

Amphenone[3] (3,3-di (*p*-aminophenyl) butanone-2 dihydrochloride) has been shown to inhibit adrenocortical steroidogenesis both *in vivo* (14) (15) and *in vitro* (16). The following study was undertaken to evaluate its effect on the adrenal medullary secretion of epinephrine and norepinephrine in response to hypoglycemia. Five animals were treated as in the preceding experiments except that 500 mg. of amphenone were administered intravenously over a two minute period 65 minutes following the injection of insulin. In addition, blood 17-hydroxycorticosteroids were determined on the adrenal venous samples obtained. No significant effect on the secretion of epinephrine and norepinephrine could be demonstrated (Table 4, Fig. 3), although a prompt but transient fall in the adrenal secretion of steroids was seen. The maximal secretion rate of 17-hydroxycorticosteroids occurred much earlier than did that of epinephrine. Indeed, in three of the five experiments the rise in steroids was maximal at 20 minutes when no significant rise in epinephrine was observed.

The epinephrine and norepinephrine concentration in peripheral venous plasma was studied in 12 experiments. There was a variable increase in the level of epinephrine over the control level in each experiment performed and the mean of the highest levels observed during hypoglycemia are illustrated in Table 5. Two animals in which extremely high levels (11 μg./L. and 16.5 μg./L.) of norepinephrine were found in the peripheral plasma have been excluded from these calculations because of pronounced muscular activity due to the occurrence of a pneumothorax in one instance and a marked lightening of anesthesia in the other. However, this increase in peripheral concentration was not associated with an unusual increase in the adrenal secretion of norepinephrine.

DISCUSSION

The resting secretory rate of the adrenal medulla of the dog has not been adequately evaluated because of the problems involved in obtaining adrenal vein blood samples without some degree of manipulation or excitement. Results of more correctly termed "control" values for the content of total catecholamines obtained by bioassay of adrenal venous blood in acutely operated, nonanesthetized (3, 17) and anesthetized (18) dogs have varied from 15 to 31 mμg./kg./min. The secretion rates from one adrenal obtained 24 hours following surgery in this study of 0.38 ± 0.47 mμg./kg./

[3] (3,3-di (p-aminophenyl) butanone-2 dihydrochloride) kindly supplied by the Upjohn Company, Kalamazoo, Michigan and Ciba Pharmaceutical Products, Incorporated, Summit, New Jersey.

TABLE 4. THE EFFECT OF AMPHENONE ON EPINEPHRINE, NOREPINEPHRINE AND BLOOD 17-HYDROXYCORTICOSTEROIDS IN DOG ADRENAL VENOUS EFFLUENT FOLLOWING INSULIN ADMINISTRATION

Exp. No.	Wt. in kg.	Hours post-op.	Substance measured[1]	Adrenal venous blood levels								
				Control periods		Experimental periods in minutes following injection of insulin.[2] Amphenone[3] injected at 65 minutes						
				20	10	20	40	60	80	95	110	125
13	20.4	1	E	0.5	0.2	0.7	4.3	12	18	24	34	
			NE	0.3	0.1	0.5	3.0	1.9	3.1	2.3	4.3	
			G		70	40	25	19	16	15	14	
			17-OHCS	3.1	5.1	33.6	11.3	10.4	10.2	7.2	15.7	
14	16.0	1	E	2.0	2.2			111	124	111	95	111
			NE	0.7	0.7			24	25	14	16	13
			G		82	48	33		25	25	29	
			17-OHCS	13.6	7.8	11.2	10.8		3.7	9.6	9.6	
15	14.5	30'	E	1.0	1.8	1.3	2.9	15	9	9	15	15
			NE	0.9	1.1	0.3	1.3	2.6	1.8	1.8	2.6	2.2
			G		77	51	33	31	33	31	20	24
			17-OHCS	25.0	26.2	36.4	37.2	35.1	10.6	16.3	11.0	17.9
16	22.2	1	E	0.9	0.2	0.6		12	12		66	
			NE	0.5	0.1	0.1		1.9	1.9		1.1*	
			G		87	50	31	24	25		25	
			17-OHCS	3.1	2.4	9.3	3.0	10.4	2.2	2.8	10.2	3.7
17	26.0	3	E	0.2	0.4	4.1	28		45	57	58	
			NE	0.2	0.2	1.8	1.1*		1.5*	1.2*	1.1*	
			G		87	52	43		52	38		
			17-OHCS	0.6	0.0	20.7	26.1	25.1	4.8	1.8	16.5	15.9

[1] E = Epinephrine mμg./kg./min. in plasma.
NE = Norepinephrine mμg./kg./min. in plasma.
G = Glucose mg. % in blood.
17-OHCS = 17-hydroxycorticosteroids in blood μg./min.
[2] Administered intravenously in dose of 1 unit/kg.
[3] Administered intravenously in dose of 500 mg.
* Inaccurate estimation of NE (see text).

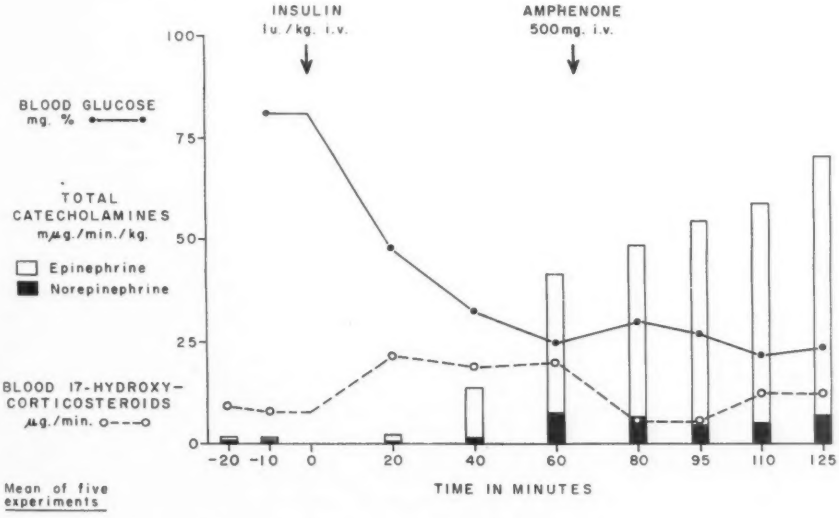

FIG. 3. The effect of amphenone on the adrenal secretion of amphenone, epinephrine and norepinephrine. The mean of values obtained in 5 experiments in Table 4 is illustrated.

TABLE 5. EFFECT OF INSULIN HYPOGLYCEMIA ON EPINEPHRINE, NOREPINEPHRINE AND TOTAL CATECHOLAMINES IN PERIPHERAL VENOUS PLASMA IN 8 DOGS STUDIED UNDER ANESTHESIA 1 TO 24 HOURS POSTOPERATIVELY

Time of sampling	Epinephrine, μg./L. ±S.D.	Norepinephrine, μg./L. ±S.D.	Total catecholamines, μg./L. ±S.D.
Before insulin[1]	0.7 ±0.46	0.9 ±0.55	1.6 ±0.91
60–120 min. after insulin	2.4 ±1.22	1.7 ±0.98	4.2 ±1.81

[1] Insulin administered intravenously in dose of 1 unit/kg.

min. for epinephrine and 0.84 ±0.92 mμg./kg./min. for norepinephrine agree closely with those reported by Lund who found less than 2 mμg./kg./min. epinephrine and less than 4 mμg./kg./min. norepinephrine in adrenal vein blood obtained from dogs under chloralose-morphine-pentobarbitol anesthesia using a less sensitive fluorometric technique (19). Aronow, using a procedure similar to that employed in this study, found secretion rates of 17 ±12.9 mμg./kg./min. for epinephrine and 3.4 ±2.6 mμg./kg./min. for norepinephrine in the adrenal vein blood of dogs that were subjected to a more extensive surgical procedure prior to the collection of samples (10). Although two of the dogs in this study had high levels of both substances 30 minutes following the operation, the levels fell very rapidly and there was no significant difference between the levels at 3 hours and at 24 hours. These very low rates of secretion are in agreement with Cannon's original observation that the adrenal medulla does not secrete continuously.

The marked and relatively rapid increase in adrenal medullary secretion occurring in response to hypoglycemia in these experiments was similar in degree and time relationship to that found by Satake in the dog (3) and Duner in the cat (6). Duner also observed a return toward control levels following the administration of glucose. Satake measured only total catecholamines while Duner estimated both epinephrine and norepinephrine and found in the cat that the increase was mainly due to enhanced epinephrine production. However, the proportion of epinephrine to norepinephrine in the adrenal secretion of the cat is lower than in the dog so that the control secretion of epinephrine constituted approximately 10% of the total amines and at the peak secretion rate was slightly greater than 50% of the total secretion. In the present study in the dog, the increase was also mainly due to epinephrine but the proportion rose from 50–88% of the total to 87–98% at peak secretion rates. In both instances norepinephrine increased modestly.

It would appear that the protective mechanism of the adrenal cortex was stimulated earlier than that of the medulla in the dog in response to hypoglycemia for in these experiments a 30 mgm.% fall in blood glucose produced an increase in 17-hydroxycorticosteroids 20 minutes after the insulin injection at a time when no significant change in epinephrine secretion had occurred.

A significant effect of amphenone on the release of epinephrine and norepinephrine from the dog adrenal medulla was not demonstrable in this series of experiments. However, a decrease in 17-hydroxycorticosteroid secretion similar to that noted by Nelson and Hume (14) and by Tullner, Graff, and Hertz (15) was observed, suggesting a specific rather than a general toxic effect on the adrenal glands.

An increase in the concentration of epinephrine and norepinephrine in peripheral plasma obtained from the femoral vein was also observed during hypoglycemia. The percentage increase in epinephrine over norepinephrine in blood from the femoral vein was not as great as that in blood coming directly from the adrenal medulla and most probably represents the addition of norepinephrine secreted peripherally at post-ganglionic sympathetic synapses. Studies in man utilizing the same method have shown a similar increase in epinephrine concentration during hypoglycemia; however, significant changes in norepinephrine were not observed (21). The values obtained are in agreement with those found in the dog by the paper chromatographic technique employed by Holzbauer and Vogt (7) and the fluorometric technique used by Millar (8).

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A THEORY FOR A QUANTITATIVE INVESTIGATION OF THE TURNOVER OF IODINE IN THE THYROID GLAND

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ABSTRACT

A theory was set up for the *in vivo* or *in vitro* investigation of the turnover of iodine in the thyroid gland using radioiodine. In this study, there was assumed to be a continuous passage into the gland of extracellular inorganic iodide and a continuous passage out, of iodinated substance(s). There were no conditions whatever placed on the nature of the intermediate metabolism, such as passage through special compartments in the gland, nor on the chemical identity of the end products. The analysis attempted to disclose whether the discharged iodinated products can be put into more than one class—each class being characterized by containing iodine which has a distinctly different rate of turnover in the thyroid gland. Assuming a steady state, the theory was further developed to determine the critical parameters of this system: the rate of passage of inorganic iodide into the thyroid, and, the mean rate of passage out, of iodine in each class.

While this theory was proposed specifically to serve as a basis for the experimental investigation of the thyroid gland, it may well apply as a method for the quantitative analysis of any general metabolic system where there is a continuous turnover of a known precursor element.

THE fact that the thyroid gland will take up and discharge radioiodine demonstrates the continuous turnover of iodine in the gland. The use of a labelling agent such as radioiodine permits a quantitative investigation of this phenomenon; but a mathematical analysis is required to interpret experimental data in terms of the rate of passage of iodine into and out of the thyroid gland.

In the past, the theoretical derivation of analyses of this kind has involved two main considerations:

(1) A mathematical expression is set up for the rate of flux of radioiodine from one hypothetical state to another in any postulated metabolic reaction, physical or chemical. It has been established in the case of phenomena independent of the isotope used (1) that, at any instant, the rate of flux of isotope from one state, where it is *uniformly distributed*, to another varies directly as the product of two factors: the rate of flux of the mass of the precursor element in the first state to the second, say r, and the ratio of the quantity of radioisotope, p, to mass, P, of labelled precursor element in the first state, i.e., the specific activity, p/P. Thus the rate of flux with

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respect to time of a radioisotope moving out of the first phase is equal to:

$$-\frac{dp}{dt} = r\,\frac{p}{P}\,. \tag{1}$$

The ratio, r/P, is actually the rate of clearance of the labelled element from this phase. By assuming either that both r and P are each constant or that r varies directly as P, the ratio r/P will remain equal to a constant which is usually called a rate constant and expressed by K. Thus,

$$-\frac{dp}{dt} = Kp. \tag{2}$$

Mathematically, this equation means that p decreases with time in an exponential manner and that the negative slope of the linear plot of ln p vs. t is equal to K.

(2) In order to apply this principle to a mathematical description of radioiodine metabolism, a simplified model of iodine metabolism has been conceived in which the total body iodine is assumed to exist in several distinct compartments and the metabolism of iodine is believed to involve the transfer of iodine from one compartment to another. Furthermore, to meet the conditions for the application of equation 2, the compartment is defined as any subdivision of total body iodine which behaves *as if it* were homogeneous (2); i.e., the introduction of radioiodine into this compartment will result in a uniform distribution. The validity of this condition is usually tested by the experimental observation that the concentration or total content of radioiodine in any compartment decreases in an exponential manner.

Currently favoured has been the hypothesis that iodine is present within the body in three compartments: 1) extrathyroidal and thyroidal inorganic iodide, 2) thyroidal organic iodine, and 3) extrathyroidal organic iodine. While in some cases free exchange between the inorganic iodide of the extracellular space and of the thyroid has been assumed (3, 4), other schemes have divided these two phases into two separate compartments (5). In any case, for the most part, it is construed that the metabolism of iodine involves the transfer of iodine from compartment 1 to 2 to 3. From this last compartment, organic iodine may be degraded whence it returns to the first. Other still more elaborate schemes have also allowed for the renal and fecal excretion of iodine belonging to the first and last compartments, respectively. And, another system envisaged the expansion in labelled iodide space which can take place early after the administration of radioiodide (8). In every case, according to equation 2, the rate of passage of radioiodine from any compartment to another would be equal to some rate constant times its content of radioiodine. Hence, a series of differential equations could be set up expressing the rate of change of radio-

iodine in each compartment as the difference between the rate of entry of radioiodine from other compartments and the rate of exit of radioiodine from that compartment.

On the basis of such compartment theories, extensive analyses of iodine metabolism have been conducted for either the whole body or for one compartment and reviewed in (2) and more recently brought up to date in the bibliographies in (6, 7, 8). The differential equations were solved either by straightforward mathematical procedures (3, 5) or by the help of an ingenious electrical analogue computer (9).

Although the introduction of the concept of a model for iodine metabolism is useful in clarifying the metabolic pathway of iodine, it nevertheless hampers the scientific reliability of any critical quantitative analysis which it serves as a basis for it is actually an *a priori* hypothesis. The significance of a rate constant expressing the flux of iodine between two compartments rests on the validity of the actual existence of those two compartments. Furthermore, as the complexity of the compartment scheme increases, the solutions for the differential equations become less unique and the possible range of values for the rate constants become wider.

Serious objections to the concept of rate constant may be raised when the compartment consists of more than one chemical type of substance. This is best exemplified in the case of the organic iodine compartment of the thyroid gland. It is an established fact that *not all* the organified radioiodine accumulated in the thyroid is, at any time, distributed in the labelled iodinated compounds which are *actually* being secreted from the gland. For instance, thyroglobulin, mono- and di-iodotyrosine are not normally released by the gland although they may contain newly-accumulated iodine (10, 11). For the development of the argument of this objection, let $y(t)$ be the total organic radioiodine contained in the gland at any time, t; similarly, let $y'(t)$ be the radioiodine contained only in those labelled iodinated substances which are actually being discharged from the gland. Also, let Y and Y' be the constant mass of iodine (I^{127}) contained in the corresponding components. Let r represent the constant rate of secretion of organic iodine from the gland. Then, it follows from equation 1, that the rate of loss of organic radioiodine from the thyroid is:

$$-\frac{dy}{dt} = -\frac{dy'}{dt} = r\,\frac{y'}{Y'}.$$

Suppose that at any time t, $y'/Y' = u(t)y/Y$, where u is a function of time. Then,

$$-\frac{dy}{dt} = u(t)\,\frac{r}{Y}\,y = Ky.$$

Now, in a given time interval, even if y decreases in an exponential manner with time (which is mathematically equivalent to a constant proportion of

the radioactivity in the gland being discharged per unit time), it only means that K is some constant which is a fraction of, equal to, or some multiple of r/Y, according to whether $u(t)$ is less than, equal to, or greater than unity. Hence, an exponential variation with time of radioactivity in a given compartment adds no critical information regarding the significance of the rate constant determined from the negative slope of the logarithmic plot. Without the guarantee that u is unity; i.e., that the specific activity of the iodine being secreted from the gland actually *equals* the specific activity of the total iodine in the gland, it follows that the rate constant, K, determined from the formula $-(dy/dt) = Ky$, is not necessarily equal to r/Y. This rule appears to have been disregarded in theory (4, 5) and in application (12).

However, a formula can be set up, to be described in this article, in which the analysis of radioiodine transfer in the thyroid gland presumes no physiological condition other than that all the iodine which enters the thyroid is as inorganic iodide coming from the plasma (through the extracellular space). No iodine compartments need be assumed *a priori* to exist within the gland and no assumption need be made concerning the chemical nature and specific activity of the labelled iodine which is being discharged from the gland. The experimental application of this theory to the quantitative investigation of the turnover of iodine in the rat thyroid is treated in the following article. It was found to be particularly useful in disclosing new concepts regarding the turnover of inorganic iodide in the thyroid gland.

THEORY

Basic Equation

At any instant, t, *following* the administration of a tracer dose of radioiodide to the body, let $[x]$ be the concentration of inorganic radioiodide in the plasma (in radioactivity units/unit vol.), while $[X]$ is the concentration of the mass of labelled inorganic iodide (I^{127}) in the plasma (mass/unit vol.). Then, $[x]/[X]$ is the specific activity. Due to a Gibbs-Donnan effect and to differences in water content, the two concentrations of inorganic iodide, isotopic and non-isotopic, may each be respectively different in the extra-cellular fluid than in the plasma; nevertheless, since each will be affected in the same manner, the ratio of one to the other, i.e., the specific activity, will be the same in the extracellular space as in the plasma.

If there be a *free* and *immediate* exchange of iodide ions between the thyroid and the extracellular space, then, an equilibrium would be rapidly established such that the specific activity of the labelled iodide in both states would be identical, i.e., equal to $[x]/[X]$. Thus, according to the principle of equation 1, the rate of entry of inorganic radioiodide into the thyroid would be equal to $[x]/[X]$ times the rate of entry of inorganic I^{127}, and, the rate of exit of inorganic radioiodide from the thyroid due to free and immediate exchange would be equal to $[x]/[X]$ times the rate of exit of

inorganic I^{127} in this process. Therefore, the *net* rate of entry of inorganic radioiodide into the thyroid is equal to $q_{in}[x]/[X]$, where q_{in} is the *net* rate of entry of inorganic iodide (I^{127}) into the thyroid (in units of mass/time) (allowing for the possible discharge of inorganic iodide by this type of exchange phenomenon). It is noted that the treatment considers only the possibility of free and immediate exchange of inorganic iodide, and, that it places no conditions on the extent to which this type of exchange may occur.

At any time, t, the rate at which radioiodine is leaving the gland can be determined by any number, n, of possible general classes of labelled iodinated substance. (The strict definition of class is developed below.) But, for each class, designated say as j, the rate is equal to the product of the rate of discharge of the labelled iodine in that class, represented by ${}_jq_{out}$, and its mean specific activity designated by ${}_j\Phi_{out}(t)$. Thus, the total rate of passage of radioiodine out of the gland, at any time, t, apart from free and immediate exchange of iodide, is equal to

$$\sum_{j=1}^{j=n} {}_jq_{out}\,{}_j\Phi_{out}(t).$$

Let y represent the content of radioiodine in the thyroid gland at any time. Then, the rate of change, with respect to time, of radioiodine in the gland can be described mathematically as the difference between the rate of entry and exit of radioiodine. Thus, the basic differential equation expressing this biological phenomenon is:

$$\frac{dy}{dt} = q_{in}\frac{[x]}{[X]} - \sum_{j=1}^{j=n} {}_jq_{out}\,{}_j\Phi_{out}. \tag{3}$$

It is noteworthy that the radioiodine content of the whole thyroid gland or of any class within it is not presumed to be uniformly distributed; so that mathematically, y, or any class thereof, is not necessarily a direct function of any ${}_j\Phi_{out}$. Therefore, equation (3) is not a first order reaction, and, for this reason, the mathematical treatment is not based on compartmental analysis.

Analysis

The nature of this analysis is first to determine whether the iodinated substance which is discharged from the thyroid gland can be divided into a number, n, (greater than unity), of classes—each class being characterized by a content of iodine which has a distinctly different rate of turnover in the gland. In order to determine experimentally whether such a class system exists, and, if so, to assess the turnover of each class, two biomathematical functions of time, each of which can be evaluated by experiment, are employed.

$$\text{Function I} = \frac{y}{\int_0^t \frac{[x]}{[X]}\,dt}. \tag{4}$$

Function I is equivalent to the ratio of the content of radioiodine in the thyroid at any time to the integral of the specific activity of the plasma iodide (the precursor of the thyroidal iodine) until that time.

$$\text{Function II} = q_{in}\frac{[x]}{[X]} - \frac{dy}{dt}. \tag{5a}$$

From inspection of equation 3, it follows that Function II is equal to

$$\sum_{j=1}^{j=n} {}_jq_{out}\,{}_j\Phi_{out},$$

which is the total rate of passage of radioiodine out of the thyroid at any time, t. For purposes of evaluation, use will be made of the formula

$$\text{Function II} = \frac{d}{dt}\left\{(q_{in} - \text{Function I})\int_0^t \frac{[x]}{[X]}\,dt\right\}, \tag{5b}$$

which can be shown to be equivalent to equation 5a.[1] (It will be shown later how q_{in} can be computed.)

The evaluation of these Functions requires the experimental administration of a tracer dose of radioiodide followed by serial determinations of the specific activity of plasma inorganic iodide $[x]/[X]$, and of the total content of radioiodine in the thyroid, y. (As such, it is compatible with *in vivo*

[1] The mathematical expansion of the right hand side of equation 5b yields:

$$\left\{\int_0^t \frac{[x]}{[X]}\,dt\right\}\frac{d}{dt}\{q_{in} - \text{Function I}\} + \{q_{in} - \text{Function I}\}\frac{d}{dt}\left\{\int_0^t \frac{[x]}{[X]}\,dt\right\},$$

that is,

$$\left\{\int_0^t \frac{[x]}{[X]}\,dt\right\}\frac{d}{dt}\{-\text{Function I}\} + \{q_{in} - \text{Function I}\}\frac{[x]}{[X]},$$

that is,

$$q_{in}\frac{[x]}{[X]} - \left\{\int_0^t \frac{[x]}{[X]}\,dt\,\frac{d}{dt}(\text{Function I}) + \text{Function I}\,\frac{[x]}{[X]}\right\}.$$

However, from equation 4:

$$y = \text{Function I}\int_0^t \frac{[x]}{[X]}\,dt$$

$$\therefore \frac{dy}{dt} = \int_0^t \frac{[x]}{[X]}\,dt\,\frac{d}{dt}(\text{Function I}) + \text{Function I}\,\frac{[x]}{[X]}.$$

Hence, the right hand side of equation 5b yields:

$$q_{in}\frac{[x]}{[X]} - \frac{dy}{dt},$$

which, according to equation 5a, is equivalent to Function II.

study.) The difficulties of this technique lie in the accurate chemical measurement of the plasma iodide, $[X]$, whether direct or indirect methods are used (4).

Following the computation of Function I for various times after radioiodine administration, the following sequence of procedure was adopted:

(1) *Determination of q_{in} from Function I*

Mathematical Rationale: The value of Function I (equation 4) in terms of q_{in} and ${}_jq_{out}$ can be derived from the integration and rearrangement of equation 3,[2]

$$\text{Function I} = q_{in} - \frac{\sum_{j=1}^{j=n} {}_jq_{out} \int_0^t {}_j\Phi_{out}dt}{\int_0^t \frac{[x]}{[X]}dt} . \tag{6}$$

Since the labelled plasma iodide (specific activity $[x]/[X]$ is considered the precursor of all the labelled thyroid iodine being discharged (specific activity ${}_j\Phi_{out}$), it follows that as $t \to 0$, the latter, ${}_j\Phi_{out}$, must approach zero before the former, $[x]/[X]$, does, so that the ratio

$$\frac{{}_j\Phi_{out}}{\frac{[x]}{[X]}}$$

approaches zero. Thus, at $t \to 0$, the ratio

$$\frac{\int_0^t {}_j\Phi_{out}dt}{\int_0^t \frac{[x]}{[X]}dt},$$

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[2] Mathematical integration of equation 3, considering q_{in} and ${}_jq_{out}$ as constant, yields:

$$\int_0^t \frac{dy}{dt}\,dt = q_{in}\int_0^t \frac{[x]}{[X]}\,dt - \sum_{j=1}^{j=n} {}_jq_{out}\int_0^t {}_j\Phi_{out}dt$$

or, since y, at $t=0$, is zero,

$$y = q_{in}\int_0^t \frac{[x]}{[X]}\,dt - \sum_{j=1}^{j=n} {}_jq_{out}\int_0^t {}_j\Phi_{out}dt.$$

Putting this latter value for y into equation 4, yields:

$$\text{Function } I = q_{in} - \frac{\sum_{j=1}^{j=n} {}_jq_{out}\int_0^t {}_j\Phi_{out}dt}{\int_0^t \frac{[x]}{[X]}dt}$$

which is equation 6.

which mathematically approaches the ratio

$$\frac{{}_j\Phi_{out}}{\frac{[x]}{[X]}},$$

itself also approaches zero. Therefore, from equation 6, as $t \rightarrow 0$, Function I $\rightarrow q_{in}$.

Hence, if a plot of Function I vs. time is extrapolated back to $t=0$, the value of Function I at $t=0$ is q_{in}. Once q_{in} has thus been evaluated, Function II may be computed by means of equation 5.

(2) *Determination of the number of classes, n, from Function II*

The interpretation of Function II (equation 5a) revealed that it represents the total rate of passage of radioiodine out of the thyroid at any time, t. Thus, in the graph of Function II vs. t, each of the classes of iodinated substances (which are being released from the thyroid with distinctly different rates of turnover of iodine in the gland) will be characterized by a peak corresponding to the time of maximum rate of release of radioiodine in this class. The times of the minima after the peaks corresponding to each class, $j=1, 2, \cdots, n$, are referred to as t_m, $m=1, 2, \cdots, n$. If the value of Function II at any t_m be negligible, then the discharge of the labelled iodine of the preceding and succeeding classes do not overlap.

(3) *Determination of ${}_jq_{out}$ from Function I*

While the iodine contained in any class may be represented by one or more different chemical substance, the rates ${}_jq_{out}$ would represent the mean rates of discharge of all the iodine in each class.

Mathematical Rationale: The quantity of radioiodide which has entered the thyroid in a time, t, is $q_{in}\int_0^t [x]/[X]dt$. Assuming a steady state such that

$$q_{in} = \sum_{j=1}^{j=n} {}_jq_{out},$$

ultimately, the quantity of radioiodine discharged in every class $j=1, 2, \cdots, n$ may be *predicted* to be equal to

$$\frac{{}_jq_{out}}{q_{in}}\left\{q_{in}\int_0^t \frac{[x]}{[X]}dt\right\}.$$

In fact, however, the *actual* quantity of radioiodine discharged in any class j is only ${}_jq_{out}\int_0^t {}_j\Phi_{out}dt$. If Function II becomes negligible in value, at $t=t_m$, then, for all $j \leq m$, the *actual* quantity of radioiodine discharged tends to be equal to the *predicted;* while for $j>m$, the actual quantity of

radioiodine discharged in these classes tends to be negligible. Accordingly, for all $j \leq m$,

$$ {}_jq_{out} \int_0^{t_m} {}_j\Phi_{out} dt = \frac{{}_jq_{out}}{q_{in}} \left\{ q_{in} \int_0^{t_m} \frac{[x]}{[X]} dt \right\}, $$

or, simplified,

$$ \int_0^{t_m} {}_j\Phi_{out} dt = \int_0^{t_m} \frac{[x]}{[X]} dt; $$

whereas, for all $j > m$,

$$ \int_0^{t_m} {}_j\Phi_{out} dt = 0. $$

Applying these results to equation 6, at $t = t_m$ Function I simplifies to

$$ q_{in} - \sum_{j=1}^{j=m} {}_jq_{out}. $$

It is also of value to note that, from equation 5b,[3]

$$ \frac{d}{at} (\text{Function I}) = (q_{in} - \text{Function I}) \frac{\dfrac{[x]}{[X]}}{\displaystyle\int_0^t \frac{[x]}{[X]} dt} - \frac{\text{Function II}}{\displaystyle\int_0^t \frac{[x]}{[X]} dt}. $$

Thus, when $t = t_m$ if Function II $= 0$, and, t_m is sufficiently great for $[x]$ to have decreased so that the product of $(q_{in} -$ Function I) and

$$ \frac{\dfrac{[x]}{[X]}}{\displaystyle\int_0^t \frac{[x]}{[X]} dt} $$

[3] By mathematical differentiation, equation 5b yields:

$$ \text{Function II} = \int_0^t \frac{[x]}{[X]} dt \frac{d}{dt} (q_{in} - \text{Function I}) + (q_{in} - \text{Function I}) \frac{d}{dt} \int_0^t \frac{[x]}{[X]} dt, $$

or,

$$ \text{Function II} = - \int_0^t \frac{[x]}{[X]} dt \frac{d}{dt} (\text{Function I}) + (q_{in} - \text{Function I}) \frac{[x]}{[X]} $$

or, rearranging, and dividing by

$$ \int_0^t \frac{[x]}{[X]} dt, $$

$$ \frac{d}{dt} (\text{Function I}) = (q_{in} - \text{Function I}) \frac{\dfrac{[x]}{[X]}}{\displaystyle\int_0^t \frac{[x]}{[X]} dt} - \frac{\text{Function II}}{\displaystyle\int_0^t \frac{[x]}{[X]} dt}. $$

is negligible, then, d/dt (Function I) $=0$.

Hence, if the graph of Function I vs. time be re-examined, at the times, t_m, the slope of the plot should be practically zero. *The value of Function I at that time is equal to*

$$q_{\text{in}} - \sum_{j=1}^{j=m} {}_jq_{\text{out}}.$$

Thus, by successive determinations at all t_m the mean rates of exit of iodine in each class, ${}_jq_{\text{out}}$ $(j=1, 2, \cdots, n)$ may be evaluated.

(4) *Determination of the specific activity functions,* ${}_j\Phi_{\text{out}}$

If the division of classes be sufficiently distinct, then there is no discharge of labelled iodine in any class $j=f$, except during the interval $t_{f-1}<t<t_f$. And, in this interval, the rate at which radioiodine is discharged from the thyroid is determined by Function II. Therefore, the ratio Function II/${}_jq_{\text{out}}$, computed at any time in the interval during which radioiodine belonging to any class j is being released, yields the mean specific activity, ${}_j\Phi_{\text{out}}$, of the labelled iodine in that class as a function of time after the administration of radioiodide.

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RATES OF PASSAGE OF IODINE INTO AND OUT OF THE THYROID GLAND OF THE RAT UNDER VARIOUS CONDITIONS OF DIETARY IODINE INTAKE AND BODY WEIGHT

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ABSTRACT

The rate of passage of iodine into and out of the thyroid gland was calculated in rats given radioiodine, on the basis of the specific activity of plasma iodide and the total radioactivity of the gland. The method, which is outlined in the previous article (2), was applied to three series of young adult rats fed graded doses of iodine and to two series of young and adult rats fed on Purina Fox Chow.

In animals receiving an adequate iodine intake a substantial fraction of the labelled iodine entering the thyroid gland is returned to the circulation within the next two or three hours, presumably in the same form as it was taken up, that is, as iodide (5). Later (20–26 hours after radioiodine administration), there is a second release of labelled iodine, presumably in the organic form. It is estimated that only 36–52% of the iodide taken up is ultimately converted into thyroid hormone, the rest being discharged as iodide.

When the *dietary intake of iodine* is reduced from a high to a low, goitrogenic, level, the fraction of the circulating iodide taken up by the gland is increased; thus, the rate of clearance of the plasma iodide rises from 0.65 to 39 ml./hour. Nevertheless, the absolute rate of entrance (q_{in}) as well as of discharge (q_{out}) of iodide are decreased. Furthermore, no change is seen in the difference between these two rates when the iodine in the diet varies from 1 to 7.5 μg per gram. This means that the rate at which iodine is retained in the gland for organic binding is not influenced by iodine intake at the levels studied. Hence under steady state conditions, that is, when the rate of secretion of hormonal iodine equals the rate of organic binding, hormone production is largely independent of iodine intake.

When the *body weight* increases without changes occurring in the iodine content of the diet, the rate of organic binding in the thyroid also increases. The variation is proportional to the total volume of thyroid epithelium and, as well, to the expected rate of oxygen consumption. From these observations, it is suggested that the size of the body conditions the volume of thyroid epithelium, which in turn controls the rate of organic binding of iodine, which then influences oxygen consumption.

THERE is evidence (1) that the main activities of the thyroid gland—uptake of iodide, organic binding of this element, and its secretion in organic combination—take place simultaneously and continuously. Furthermore, these activities occur at a constant rate or, at least, at a mean rate over any limited period of time. By a method described elsewhere (2), the rates of these activities can be determined and expressed in absolute terms, that is, as mass of iodine involved per unit of time. The method was

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applied to young and adult rats fed on a Purina diet, as well as to 3 groups of pubescent male rats fed on diets containing very low, medium, and high amounts of iodine respectively.

METHODS

Experimental animals

The series of experiments conducted to test the effect of graded doses of dietary iodine on the thyroidal metabolism of iodine included three sets of albino rats, with average body weights of 151, 129 and 146 (± 5) gm. which, for twenty days prior to the injection of radioiodide, were fed respectively: Remington's low-iodine diet (set I), the same diet to which 1 μg. iodide per gram of diet was added by spraying a known quantity of diet with a known volume of a solution of sodium iodide prior to mixing (set II), and Remington's diet with a similarly added supplement of 7.5 μg. iodide per gm. of diet (set III).

In the second series, two sets (IV and V) of male hooded rats weighing an average of 58 and 259 gm. respectively were fed on Purina Fox Chow (Canadian brand, estimated to contain about 2 μg. of iodine per gm. of diet).

Each set of rats was subdivided into seven groups of six animals each, which were kept in cages with screened flooring to prevent access to excreta. Measurements of the food intake revealed that the daily ingestion of iodine was approximately <1, 10 and 75 μg. for sets I, II and III respectively, and 10 and 20 μg. for sets IV and V respectively.

Each of the rats in sets I, II and III received ten microcuries of radioiodide, I^{131}, intraperitoneally, those of set IV, five microcuries subcutaneously, and those of set V, twenty microcuries subcutaneously. In each set, a group of rats was sacrificed at each of the following times after I^{131} injection: 1, 4, 10, 24, 48, 96 and 192 hours (except for set I in which the groups were sacrificed at 1, 3, 7, 12, 30, 40 and 72 hours). The injections and sacrifices were arranged to fall during daylight hours, when the rats being nocturnal in habit feed little or not at all. In this way, the likelihood of a sudden increase in circulating iodide due to food ingestion was decreased. Each animal was anesthetized with ether and blood was withdrawn by aortic puncture into a heparinized syringe. In the first series, the whole thyroid of every animal was used for chemical and radioactivity analysis. In the second series, the left lobe only was so used and the results obtained were doubled to give values comparable to those of the first series; meanwhile the right lobe was fixed in Bouin solution and then stained with the periodic acid-Schiff-haematoxylin technique for histological study.

Iodine analysis: chemical and radioactive

Plasma. The plasma of each animal was separated by centrifugation and divided into two one ml. samples.

The first one ml. sample was used for quantitative determination of inorganic iodine (I^{127}). This was performed individually on all of the 42 samples in set V, on the pooled 42 samples in each of sets I, II and III, and on 14 combinations of three samples (from the same group) in set IV. In sets I, II and III, the inorganic iodide was separated by zinc sulfate precipitation and estimated by a dry-ashing prodecure modified from Barker's method (3) by H. Isler in this Department, while in sets IV and V the inorganic iodide was separated by trichloracetic acid (TCA) and estimated according to a modified Chaney procedure (4). The results were expressed as the mean concentration of inorganic iodide (I^{127}), that is, in μg. per ml. of plasma and referred to by the symbol $[X]$ in Table 1. In the case of set I, the concentration of plasma iodide was too low for detection, but, in view of the known sensitivity of the method, it was possible to state that the amount of iodide present was less than 0.01 μg. per ml.

TABLE 1. EXPERIMENTAL DATA

Animal set (diet)	Body weight	Plasma concentration of inorganic iodide $[X]$	Time after I^{131} injection t	Plasma concentration of inorganic radioiodide $[x]$	Total radioiodine in thyroid y
	gm.	μg./cc.	hours	10^3cts./100sec./cc.	10^3cts./100sec.
I (Remington)	151	<0.01	1	0.440	44.9
			3	0.192	56.6
			7	0.046	79.1
			12	0.025	54.9
			20	—	31.0
			40	—	30.0
			72	—	19.6
II (Remington + 1 μg. I/gm.)	129	0.056	1	2.180	3.10
			4	0.755	6.54
			10	0.366	8.84
			24	0.081	12.00
			48	0.019	8.03
			96	—	4.60
			192	—	1.94
III (Remington + 7.5 μg. I/gm.)	146	0.169	1	2.09	1.42
			4	1.37	2.77
			10	0.50	4.41
			24	0.138	4.60
			48	0.025	3.89
			96	—	1.60
			192	—	0.87
IV (Purina) (Young)	58	0.179	1	2.12	1.08
			4	1.21	2.80
			10	0.42	3.90
			24	0.06	3.85
			48	—	3.13
			96	—	2.08
			192	—	1.00
V (Purina) (Adult)	259	0.104	1	2.30	3.8
			4	1.31	8.2
			10	0.51	10.0
			24	0.07	11.8
			48	—	9.7
			96	—	7.0
			192	—	4.8

The second sample was used for the measurement of inorganic radioiodide (I^{131}). In every case, the inorganic radioiodide was obtained as the TCA soluble fraction by adding an equal volume of cold 10% TCA to the sample, shaking, centrifuging, and, finally repeating this procedure twice on the precipitate with a double volume of cold 5% TCA. The total supernatant was made up to 10 cc. with 4N sodium hydroxide, dried at 35° C, and counted by an end window beta ray counter. The mean results per groups of 6 rats were recorded under the symbol $[x]$ in Table 1 and plotted as the logarithm of the concentration of inorganic radioiodide per ml. of plasma vs. time after administration of I^{131}. The graphs were then extrapolated back to time zero.

Thyroid. Each thyroid sample was digested in 9 ml. of 2.2N sodium hydroxide and then made up to 10 ml. with a 30% solution of TCA. The concentration of 2.2N sodium hydroxide and 30% TCA in these solutions were calculated so that the final 10 ml. volume would contain about the same mass of sodium hydroxide and TCA as in the 10 ml. volume of plasma used for radioactivity measurement as described above. Three

one ml. samples of each solution were plated for the measurement of thyroid radioactivity. The mean radioiodine content of the glands at various time intervals after I^{131} administration were listed in arbitrary units (counts/100 sec.) for purposes of calculation (symbol y, Table 1) and in per cent injected dose for purposes of discussion.

Biomathematical analysis

The experimental data for each of the five sets of rats were analyzed by the method proposed in the preceding article to estimate the entry and exit of thyroidal iodine (2). For this purpose, it was necessary to solve two mathematical expressions referred to as Functions I and II, namely,

$$\text{Function I (Fn I):} \quad \frac{y}{\int_0^t \frac{[x]}{[X]}\,dt}$$

$$\text{Function II:} \quad q_{in}\frac{[x]}{[X]} - \frac{dy}{dt}$$

which may be shown to be equivalent to:

$$\frac{d}{dt}\left\{q_{in} - \text{Fn I}\right\}\int_0^t \frac{[x]}{[X]}\,dt$$

The symbols used are listed below.

Experimental data (Table 1):

$[x]$ stands for the concentration of inorganic radioiodide in plasma (in counts/100 sec./ml.),

$[X]$, the concentration of inorganic iodide in plasma (μg./ml.),

y, the content of radioiodine in the thyroid (in counts/100 sec.),

t, the time after injection of radioiodine (in hrs.).

Calculated data (Table 2):

q_{in}, the rate of passage of iodide into the thyroid gland (μg./hr.),

${}_jq_{out}$, the rate of passage of iodine out of the thyroid gland (μg./hr.). The left subscript $j(=1, 2, \cdots, n)$ indicates the possibility that a number n, of separate classes of iodine with distinct turnover rates may be released from the gland.

The variable factors in the analysis are $[x]$ and y. The quantitative value of the plasma radioiodide concentration at various times was used for $[x]$, it being immaterial that the breakdown of labelled thyroid hormone adds some radioiodide to the plasma at later intervals. The number of experimental points for $[x]$ and y was estimated, on the basis of previous experience, to be large enough to allow extrapolation safely.

For each set of animals examined, the procedure adopted was as follows:

(1) The denominator of Function I, that is, the integral with respect to time of the specific activity of plasma iodide, $\int_0^t \frac{[x]}{[X]}\,dt$, was calculated for various times t_i (e.g. $t_i = 1, 2, 4, 6, 8, 10, \cdots$, hours).[1] The numerator of Function I was obtained directly

[1] This integration was accomplished by assuming that within each selected time interval $t_{i-1} \rightarrow t_i$, $[x]$ decreased exponentially while $[X]$ remained constant. Thus,

$$\frac{[x(t)]}{[X]} = \frac{[x(t_{i-1})]\,e^{-s_i(t-t_{i-1})}}{[X]}, \qquad t_{i-1} \leqslant t \leqslant t_i;$$

therefore,

$$\int_0^{t_i} \frac{[x]}{[X]}\,dt = \sum_0^i \frac{[x(t_{i-1})] - [x(t_i)]}{s_i[X]}.$$

The factor s_i was calculated from the negative slopes of the linear plot $\ln[x]$ vs. t. The time intervals were selected in such a way that in each case the slope of the curve of $\ln[x]$ vs. t approximated linearity.

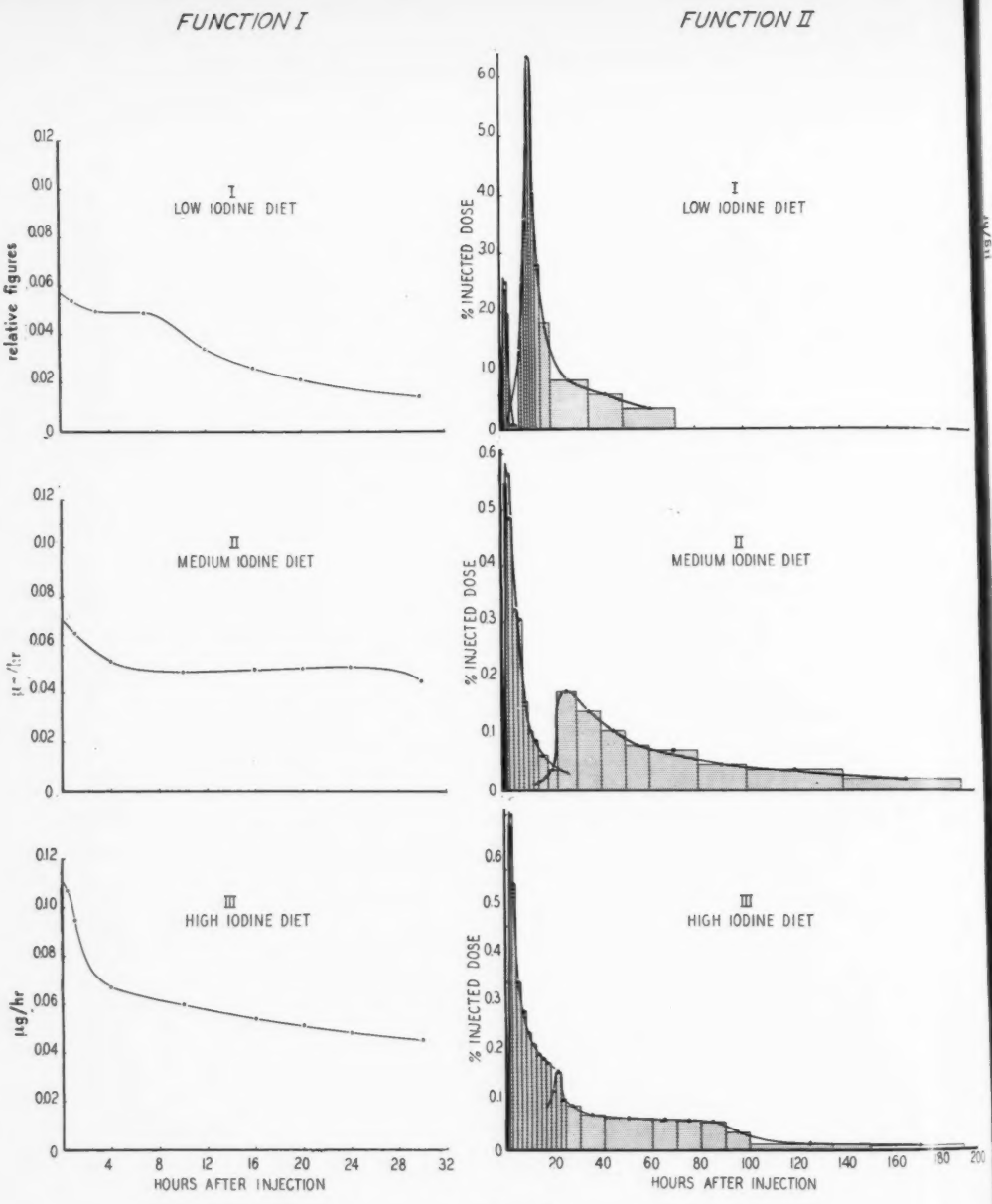

PLATE I

FIGS. 1, 3 AND 5. *Functions I of Sets I, II, and III, from the top down on the left*

Plots of Function I vs. time after administration of radioiodine to rats of about the same weight maintained on Remington's low iodine diet (set I, fig. 1), on the same diet plus 1 μg. of iodine per gm. (set II, fig. 3) or plus 7.5 μg. of iodine per gm. (set III, fig. 5). The dimensions of Function I are μg. iodine per hour in Figs. 3 and 5; but relative figures had to be used in Fig. 1, since in set I the plasma concentration of iodide was too low to be measured and thus no absolute values could be calculated.

Extrapolated back to $t=0$, Function I yielded the mean rate of entry of inorganic

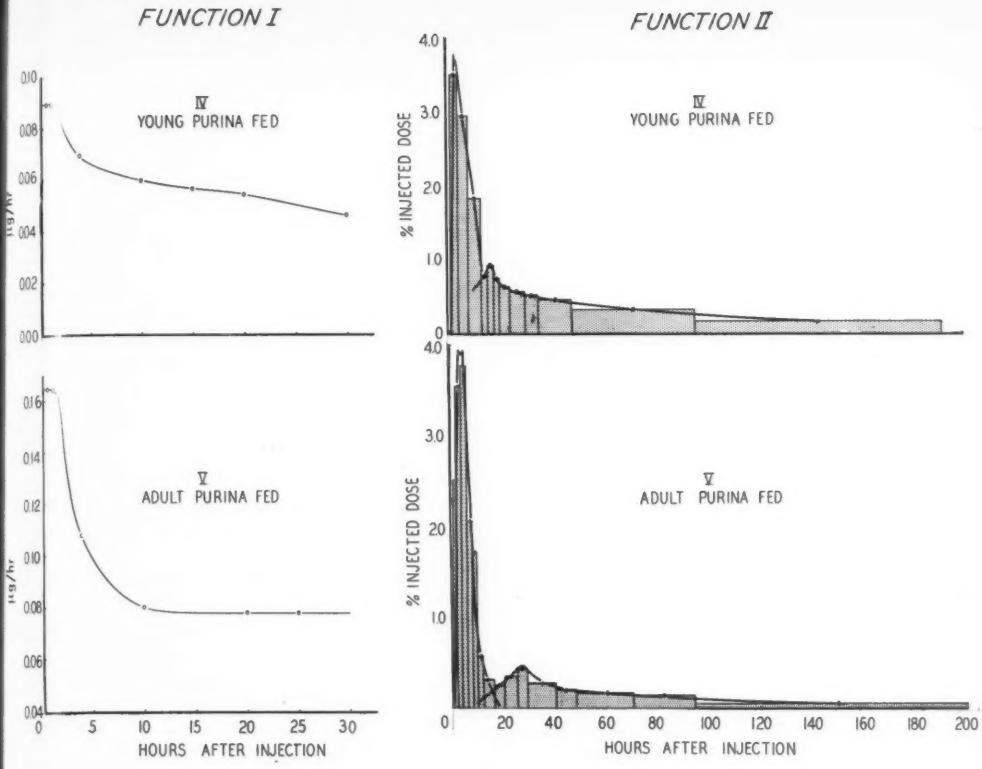

PLATE II

FIGS. 7 AND 9: *Functions I of Sets IV and V, from the top down on the left*

Plots of Function I vs. time after administration of radioiodine to young (set IV, Fig. 7) and adult rats (set V, Fig. 9).

FIGS. 8 AND 10: *Functions II of Sets IV and V, from the top down on the right*

Plots of Function II vs. time after administration of radioiodine to the animals of sets IV (Fig. 8) and V (Fig. 10). The two peaks indicate the existence of two classes of radioiodine released from the thyroid with different turnover times.

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iodide, q_{in}. As Function I first levelled off (and Function II was at a minimum, that is, at 5 and 20 hours for sets I and II, respectively), the value of Function I yielded ($q_{in} - {}_1q_{out}$). In the high iodine diet set (set III), there was no distinct levelling off of Function I, and the estimation of ($q_{in} - {}_1q_{out}$) which was based on the suggested minimum in Function II was less accurate (see footnote 4).

FIGS. 2, 4 AND 6: *Functions II of Sets I, II, and III, from the top down on the right*

Plots of Function II vs. time in sets I (Fig. 2), II (Fig. 4) and III (Fig. 6). Function II indicates the rate at which newly entered radioiodine was being discharged from the thyroid gland. The absolute units are of little significance since they were dependent on experimental conditions. Except for the rats of set I kept on a low iodine diet (Fig. 2), the curves showed two peaks, which indicated the existence of two classes of radioiodine released with different turnover times, each class being characterized by a separate peak. Even in set I, the substantial early release of radioiodine (with a peak at less than 3 hours after administration of radioiodine) means that some radioiodine came out of the thyroid with a relatively short turnover time.

TABLE 2. RATES OF PASSAGE OF IODINE INTO AND OUT OF THE THYROID GLAND

Animal set (diet)	Body weight	Approximate iodine dietary intake	$[X]$ Plasma concentration of inorganic iodide	q_{in} Rate of passage of iodide into the gland	${}_1q_{out}$ Rate of discharge of class 1 iodine (Rate of iodine discharge)	$q_{in} - {}_1q_{out}$ Rate difference (Rate of iodide retention)	$q_{in}/[X]$ Rate of clearance of plasma iodide	${}_1q_{out}/q_{in}$ Fraction of the entering iodide discharged as class 1 iodine
	gm.	μg./day	μg./ml.	μg./hr.	μg./hr.	μg./hr.	ml./hr.	%
I (Remington diet)	151	1	<0.01	—	—	—	39	14
II (Remington diet +1 μg. I/gm.)	129	10	0.056	0.070	0.020	0.050	1.2	29
III (Remington diet + 7.5 μg. I/gm.)	146	75	0.169	0.110	0.060	0.050	0.65	56
IV-Young (Purina)	58	10	0.179	0.090	0.058	0.032	0.50	64
V-Adult (Purina)	259	20	0.104	0.164	0.078	0.086	1.6	48

from experimental data (Table 1, last three columns) or from extrapolated values of these data plotted vs. t. Graphs of the various Functions I were then drawn (Figs. 3, 5, 7 and 9). In the case of set I, since $[X]$ was not determined, Fn $I/[X]$ which is equal to $y/\int_0^t [x]dt$ was evaluated instead (Fig. 1).

The rate of iodide entry into the thyroid, q_{in}, was assumed to be equal to the value of Function I at $t=0$ (see previous article) and therefore was obtained by extrapolation (Figs. 3, 5, 7, 9 and Table 2). In the case of set I, Function I was not obtained in absolute terms; however, $q_{in}/[X]$ (equal to Fn $I/[X]$ at $t=0$) could be determined and was reported in Table 2, column 7 under the heading "rate of clearance of plasma iodide."

(2) To determine Function II, it was first necessary to obtain Fn I, q_{in} and $\int_0^t \frac{[x]}{[X]}\,dt$, according to the above procedure. The specific activity of the plasma iodide, $[x]/[X]$, was derived from experimental data (Table 1). The factor $\frac{d\ \text{Fn}\ I}{dt}$ was estimated over any given short time interval $t_i \leqslant t \leqslant t_{i+1}$ by the formula

$$\frac{\text{Fn I}(t_{i+1}) - \text{Fn I}(t_i)}{t_{i+1} - t_i}$$

In the case of set I, Function II was computed by using

$$\left(\frac{q_{in}}{[X]} - \frac{\text{Fn I}}{[X]}\right)[x] - \frac{d\ \text{Fn I}}{dt}\ \frac{1}{[X]} \int_0^t [x]\,dt$$

which is the same formula rearranged to allow for its evaluation without knowledge of $[X]$ itself. Block diagrams were constructed for Function II vs. t (Figs. 2, 4, 6, 8 and 10).

RESULTS AND INTERPRETATION

Function II

According to theory (2), *Function II represents the rate at which the newly-entered radioiodine passes out of the thyroid gland.* Inspection of the graphs of this Function vs. time (Figs. 2, 4, 6, 8 and 10) indicated that, in every case, there was a release of radioiodine from the thyroid gland at a rate which began at zero, rose to a maximum in about 2 hours, and then decreased steadily. A distinct second peak was observed in sets I, II and V

respectively at 10, 22, and 26 hours after radioiodine administration. In sets III and IV, examination of the graphs suggested that there might also be a second peak at 20 and 21 hours respectively. It was concluded that in sets I, II and V (and probably III and IV), the labelled iodine was released from the thyroid gland in two separate classes. From the differences in the times at which the two classes are released, it is inferred that, at least in sets I, II and V, each class consists of iodine having a distinctly different rate of turnover in the gland.[2]

The chemical nature of iodine in these two classes was not obvious. It was, therefore, decided to refer to the rapidly metabolized labelled iodine as class 1 iodine and to the slowly metabolized labelled iodine as class 2 iodine. Comparison of the graphs of the various Functions II indicates that the second peak (and probably also the first) is closer to the time origin in set I (10 hrs.) than II (22 hrs.) and III (22 hrs.) and in set IV (21 hrs.) than V (26 hrs.) Figs. 2, 4, 6, 8 and 10). It is, therefore, concluded that, in general, the turnover rates of both classes of iodine were reduced by high dietary iodine intake (sets I–III) and with increasing body weight (sets IV–V).

Function I

The mean absolute rate, q_{in}, at which iodide was taken up by the thyroid gland in the animals of sets II–V was determined (2) by extrapolating to time 0 the graphs of Function I vs. time (Figs. 3, 5, 7 and 9). In this manner it was found (Table 2) that the rate of iodide uptake increases with the iodine content of the diet (sets II–III) and with age (sets IV–V). However, the values for the clearance of plasma iodide, $q_{in}/[X]$ (Table 2), indicated that the clearance varied inversely with the iodine content of the diet in sets I, II and III.

The measurement of the rate, ${}_1q_{out}$, at which class 1 iodine is released by the thyroid gland required several steps. It was first noted that, when the first peak in Function II graphs (Figs. 2, 4, 6, 8 and 10) indicated the release of this iodine, there was a steady decrease in the value of the corresponding Functions I (Figs. 1, 3, 5, 7 and 9). In fact, when the slope of the graph for Function I approached zero, that is, at approximately 5, 20, 19, 14 and 18 hrs. for sets I–V respectively, the value of Function II, as predicted by theory (2), reached a minimum.[2] This observation indicated that the turnover of class 1 iodine was then practically complete. At the time of this minimum, theory predicted that the value of Function I should be equal to $q_{in} - {}_1q_{out}$, that is, the difference between the rate of up-

[2] In sets III and IV, the minimum in Function II was not distinct, neither did the slope of Function I vs. time quite reach zero. This was attributed to the fact that, due to the relatively high iodine feedings, the turnover rates of the two classes of iodine were not sufficiently distinct.

take and discharge of iodide (rate of retention of iodide). Calculation of this difference for sets II–V (Table 2) revealed that it was not influenced by the level of dietary iodide (sets II–III), but increased with the body weight (sets IV–V).

The mean rate of discharge of class 1 iodine from the thyroid gland, ${}_1q_{out}$, was obtained by subtracting $(q_{in} - {}_1q_{out})$ from q_{in} (Table 2, 6th column). This rate varied directly as the level of iodine in the diet (sets II–III) and as the body weight (sets IV–V). Finally, the percentage of the iodide taken up which is released as class 1 iodine, ${}_1q_{out}/q_{in}$, (Table 2, last column) varied as the concentration of inorganic iodide in plasma, $[X]$.

In the case of set I, the value of ${}_1q_{out}$ could not be calculated in absolute terms, but the value of Fn I/$[X]$ was used at time $t=0$ to measure $q_{in}/[X]$ and, at the time when Function II was minimum, to measure ${}_1q_{out}/[X]$. Thus, the ratio ${}_1q_{out}/q_{in}$ was computed. Accordingly the clearance of plasma iodide and the percentage of the iodide taken up which is released as class 1 iodine could be calculated in this set (Table 2).

Since the animals investigated in each set were maintained on their respective diets for twenty days prior to the administration of radioiodide, it was presumed that the thyroid glands had reached a condition of steady state with regard to their iodine metabolism. Thus, the rates reported in Table 2 describe steady states for the thyroid glands of rats of a given body weight and a given dietary level of iodine, kept under similar environmental conditions.

DISCUSSION

The uptake of inorganic iodide

The selective entry of inorganic iodide into the thyroid gland is the first step in the formation of thyroid hormone. Iodide is made available to the thyroid follicles from two sources: an external, the extracellular fluid, and an internal one, the deiodination of organic compounds within the gland. It is presumed (5) that iodide ions are extracted from the extracellular fluid by the cells enclosing the follicle, whereas, once iodide ions are within the follicle, they are retained in, and by, the colloid (6).

In the analysis reported in this article, q_{in} represented the rate of uptake of extrathyroidal inorganic iodide by thyroid glands in a steady state. As indicated by the values for this rate (Table 2), the amounts of iodide taken up by the gland increased with age (0.090 and 0.164 μg./hr. in 58 and 259 gm. Purina fed rats respectively) presumably due to the concomitant increase in thyroid size, and increased as well with the dietary intake of iodine (0.070 and 0.110 μg./hr. in rats of about the same weight fed diets containing 1 and 7.5 μg. per gram respectively).

However, the ratio $q_{in}/[X]$, that is, the rate of clearance of inorganic iodide by the gland, was probably a truer index of the capacity of the thyroid

to concentrate iodide from plasma. Such clearances were inversely proportional to the level of plasma (or dietary) inorganic iodide (Table 2; sets I–III). The clearance was particularly high in the animals fed a low level of iodide (set I), in which it is known that the thyroid gland is hyperactive, as demonstrated by histological criteria (tall columnar epithelium, thin colloid, vascularity).

The nature of the iodine discharged as class 1 and class 2

Following the administration of a single dose of radioiodide to Purina-fed rats, it had been shown that there was no organic iodine released from the thyroid by 3 hours after injection, while a significant release was taking place by 24 hours (7). Accordingly, it was concluded that the rapidly metabolized labelled iodine in class 1 (represented by the first peak in the graphs of Function II occurring about 2 hours after administration) did not include organic iodine, and hence could only be identified as inorganic iodide. Thus the thyroid gland discharged as such some of the iodide which had just been taken up. (The findings on the rate of discharge of class 1 iodine, ${}_1q_{out}$, as reported in Table 2, must therefore be interpreted as referring to the discharge of iodide.) On the other hand, class 2 iodine (represented by the second peak of Function II) was essentially due to organic iodine.

The discharge of inorganic iodide

Until recently it was generally assumed that, under normal conditions, the inorganic iodide taken up by the gland was completely converted into organically bound iodine. Accordingly the rate of iodide entry was believed to equal the rate of organic binding and the rate of hormonal iodine output, so that the iodide concentrating capacity of the gland would determine the rate of hormone formation (8, 9). It was further thought that iodide could only be discharged from the thyroid gland in cases of total inhibition of protein binding (8).

However, four years ago, it was reported in a preliminary form, that in the rat some of the iodide taken up by the thyroid gland was soon discharged (5). Brown-Grant *et al.* (10) reported a release of radioiodine from the rabbit thyroid soon after administration of I^{131}. This released iodine, which they presumed to be organic, may well have been in the inorganic form. Later, Ingbar and Freinkel, also in the rat (11), found that there is a gradient of thyroidal to plasma iodide, which is markedly diminished by perchlorate administration. They concluded from these results that iodide may be discharged from the thyroid, even when it is not under the influence of agents blocking organification. Hickey and Brownell (12) in man observed a higher level of urinary radioiodide than expected from data provided by the analogue computer and speculated that this iodide was of thyroid origin. The following year, Ingbar (13) queried whether all of the io-

dide taken up by the thyroid in man was subsequently bound to protein. Still more recently, application of the analysis reported in this article to several euthyroid young adult males confirmed that there is a discharge of inorganic iodide from the human thyroid (unpublished work with M. Cunningham and H. Isler).

The work in the rat here presented indicated that a fraction of the iodide taken up by the gland is released as such to the extrathyroidal space and, therefore, is not used for hormone synthesis. The percentage of the newly-entering iodide which is discharged

$$\frac{{}_1q_{out}}{q_{in}} \times 100$$

was only 14% in rats fed the low iodine diet but rose to 48–64% in rats maintained on large amounts of dietary iodine (Table 2, sets III–V).

The difference between the rates of iodide uptake, q_{in}, and discharge, ${}_1q_{out}$, that is $q_{in} - {}_1q_{out}$, is equivalent to the net rate of retention of iodide. Under conditions of steady state with a constant content of inorganic iodide in the gland,[3] whatever iodide is retained must be bound into protein and so $q_{in} - {}_1q_{out}$ must also designate the net rate of organic binding.

When the rate of iodide uptake, q_{in}, and the net rate of organic binding are compared (Table 2), there is apparently no simple relation between them. This finding invalidates current concepts that the rate of iodide uptake equals the rate of organic binding (1) or that the iodide concentrating capacity of the gland determines the rate of hormone formation (8, 9).

Prior to assessing what relation, if any, exists between iodide uptake and efficiency of organic binding, it should first be pointed out that not all of the iodide discharged by the thyroid need be derived solely from newly entered iodide. In fact, Roche and Michel (14) have shown that some of the thyroid iodide arises as the result of deiodination of organic compounds within the gland. Since presumably both the newly entered iodide and that derived from deiodination mix freely in a common 'pool' (probably located in the colloid of the follicles), those iodide ions discharged from the gland must also originate from both sources and, hence, represent that portion of the iodide made available to the gland which escapes organic binding. Per unit time, only a fraction, λ, of the pooled iodide will undergo protein binding. So far in this paper the rate at which iodide is taken up by the gland from the extracellular space has been called q_{in}. The rate at which iodide is provided by deiodination may be called d. Then, the rate of discharge of inorganic iodide from the gland, ${}_1q_{out}$, will be the difference between the

[3] From the data presented here, it was deduced that the fraction of the thyroidal iodine present as inorganic iodide, in a steady state, would be of the approximate order of 5% (21).

total rate at which iodide is made available to the thyroid, $(q_{in}+d)$, and the rate at which it is organified, $\lambda(q_{in}+d)$, i.e.,

$$ {}_1q_{out} = (q_{in} + d) - \lambda(q_{in} + d) = (1 - \lambda)(q_{in} + d). \quad (1) $$

It is currently believed that the organic binding of iodine involves a sequence of reactions within the thyroglobulin molecule leading to the formation of mono- and di-iodotyrosine, the coupling of which forms tri-iodothyronine and thyroxine. Simultaneously with this process, there is a continuous proteolysis of thyroglobulin yielding the free iodinated amino-acids, of which only two, thyroxine and tri-iodothyronine, have been found in the plasma (presumably by excretion thereto) (7), while the remaining amino-acids, mono- and di-iodotyrosine, are deiodinated within the thyroid (14). Now, if the ratio of the thyroglobulin iodine bound into tyrosyl radicals to that bound into thyronil radicals is n (within wide limits n is of the order of 2) (15), the rate, d, at which iodide is made available by deiodination would be expected to be n times that at which iodine is secreted as hormone. Since the latter in a steady state is equal to the net rate of binding of iodine to protein, then $d = n(q_{in} - {}_1q_{out})$. Hence the total rate at which iodide arises in the gland is:

$$ q_{in} + d = q_{in} + n(q_{in} - {}_1q_{out}) = (n + 1)q_{in} - n{}_1q_{out}. $$

Putting this into equation (1):

$$ {}_1q_{out} = (1 - \lambda)\{(n + 1)q_{in} - n{}_1q_{out}\} $$

and, therefore:

$$ \lambda = \frac{1 - \dfrac{{}_1q_{out}}{q_{in}}}{1 - \dfrac{n}{n+1}\dfrac{{}_1q_{out}}{q_{in}}}. $$

It is deduced from this that λ varies inversely as ${}_1q_{out}/q_{in}$.

The values of λ worked out for the various experiments assuming $n = 2$ were respectively 0.95, 0.88 and 0.70 for sets I–III, and 0.63 and 0.76 for sets IV–V. Thus the efficiency of organic binding, as measured by the steady state value of λ varied inversely as the plasma concentration of inorganic iodide.

From this theory, it is possible to predict the proportion of the discharged iodide which is derived from deiodination of organic compounds within the gland. This is simply $(1-\lambda)d/{}_1q_{out}$, which is

$$ \frac{(1 - \lambda)n(q_{in} - {}_1q_{out})}{{}_1q_{out}}, $$

which reduces to

$$\frac{1 - \frac{{}_1q_{out}}{q_{in}}}{\frac{n+1}{n} - \frac{{}_1q_{out}}{q_{in}}}.$$

Again, assuming as above that $n=2$, and, putting in the values determined for ${}_1q_{out}/q_{in}$, this proportion turned out to be 0.63, 0.59, 0.47 for sets I–III, and 0.42 and 0.51 for sets IV–V. Hence, approximately half of the iodide discharged from the thyroid gland was estimated to originate from the deiodination of organic compounds.

The organic binding of iodine

It was shown above that the net rate of accumulation of organic iodine in the gland is equal to $q_{in} - {}_1q_{out}$ (Table 2). However, this value does not necessarily represent the rate of all the binding of iodine to protein. It was indicated that the rate at which iodide was made available to the gland was actually $q_{in}+d$, where d is the rate at which inorganic iodide is liberated in the thyroid due to deiodination of organic compounds. Hence, the rate of *total* protein binding of iodine in the thyroid is more precisely, $q_{in}+d-{}_1q_{out}$. Assuming as indicated above that d might be of the order of $n(q_{in}-{}_1q_{out})$, then *the rate of total protein binding of iodine is* $(n+1)(q_{in}-{}_1q_{out})$. From these formulae, it is concluded (Table 2; sets II and III) that where the rate of *net* retention was 0.050 μg. of iodine per hr., that of *total* binding to protein was $(n+1)$ times 0.050 μg. per hour and that both these rates were the same in animals fed medium or high dietary levels of iodine, provided n remained the same. Putting n equal to 2 as above then, the rate for total organic binding was 0.150 μg./hr.

The similarity of the rate of organic binding in animals fed medium or high dietary iodine supports the contention (1) that, in the rat, despite fluctuations in the amount of iodide ingested and in the physiological activities of the animal, the *steady state* rate of organic binding and secretion is constant. No doubt, there must be one or more regulatory mechanisms by which this steady state may be secured. Thus the level of circulating thyroid hormone exerts some control on the rate of release of the thyrotrophic hormone by the anterior pituitary. More recently, it has been proposed that inherent in the thyroid gland itself is a partially autonomous capacity for the regulation of the rate of concentration of inorganic iodide (16, 17).

It may be possible to relate the rate of organification of iodide to structural conditions. In a recent article, the authors presented evidence that the rate of accumulation of organic iodine in any follicle is dependent directly upon the mass of its epithelial cells (18). By generalizing, it was

suggested that the rate of accumulation of organic iodine by the whole thyroid gland was governed by the epithelial mass, that is, the aggregate mass of the cells of all follicles.

Now the total rate of accumulation of organic iodine has been shown above to be $(n+1)(q_{in}-{}_1q_{out})$. Using the value of q_{in} reported in Table 2 for the young and adult rats on Purina diet (sets IV and V) the rates of organification are $(n+1)0.032$ and $(n+1)0.086$ μg/hr., respectively. Employing a histometric technique reported in detail in the literature (19), the mean total epithelial mass in the thyroids of the young and adult rats used in these experiments was estimated as 2.07 and 5.69 mg. respectively.[4] The ratio of the rate of organification to epithelial mass for each set was, therefore $(n+1)0.0155$ and $(n+1)0.0151$ μg./hr./mg.—two values which are practically identical.

The above calculations support the previous conclusion (18) that the rate of organification of iodine by the thyroid follicle is quantitatively governed by its epithelial mass.

The discharge of organic iodine

Since following radioiodine injection to Purina fed rats, organic iodine had appeared in the circulation by 24 hours after injection (7), it is concluded that most of the slowly metabolized iodine released as class 2 iodine consisted of organic radioiodine, although some inorganic iodine must also be present.

It has been pointed out that, under steady state conditions, the secretion rate of class 2 iodine is equal to the net rate of retention of iodide, $q_{in}-q_{out}$. Thus, for the young (set IV, 58 gm.), young adult (average sets I–III, 142 gm.), and adult (set V, 259 gm.) rats investigated, it was 0.032, 0.050 and 0.086 μg./hr. respectively, which, if the fraction of class 2 present as iodide is disregarded, would be equivalent to L-thyroxine secretion rates of 1.2, 1.8, and 3.2 μg. per day. These results compare favorably with those estimated for the rat by other methods (20).

Per body weight (B.W.), these values for the rates of organification of iodine—which are also assumed to be the rates of secretion of class 2 iodine—are greater for the smaller animals (Table 3), but, per $B.W.^{2/3}$, they are almost exactly identical in all the groups (Table 3). The interest in this comparison, of course, comes from the fact that one of the characteristic effects of thyroid hormone is the maintenance of metabolic rate, the value of which is known to be related in animals of different weights to $B.W.^{2/3}$.

[4] This technique consisted of making large scale projections of thyroid sections and drawing numerous parallel lines between the outer boundaries of the gland. The mean ratio of the total length of all the intercepts across epithelial tissue of the follicles to the total length of all the drawn lines was taken to be equivalent to the ratio of epithelial mass to glandular mass. By multiplying this ratio by the thyroid weight, a value for the epithelial mass was obtained.

TABLE 3. SECRETION RATE OF ORGANIC IODINE BY THE THYROID

Animal set (Diet)	Body weight (B.W.) (gm.)	Rate of secretion of organic iodine		
		μg./hour ($q_{in} - {}_1q_{out}$)	μg./hour per gm. B.W. $\times 10^3$	μg./hour per gm. B.W.$^{2/3}$ $\times 10^3$
IV (Purina)	58	0.032	0.55	2.1
II–III (Remington diet—iodine)	142	0.050	0.35	1.9
V (Purina)	259	0.086	0.33	2.1

These results suggest, therefore, that in the growing animal, the increase in total oxygen consumption is due directly to the rate of protein binding of iodine by the thyroid, which, in turn, is related to the increase in volume of thyroid follicle epithelium.

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THE EFFECT OF ANTERIOR HYPOTHALAMIC LESIONS ON THYROID FUNCTION AND GOITER DEVELOPMENT IN THE RAT[1]

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ABSTRACT

Thyroid function was measured in rats lesioned in the anterior hypothalamus, using the 24-hour radioiodine uptake test, the hormone discharge rate both on a control diet and during the administration of propylthiouracil, the T/S ratio test of VanderLaan, the PBI and the serum cholesterol level. The results of all tests with the exception of the T/S ratio indicate an interference with hypophyseal thyrotropin production in lesioned animals, with lesions which block the hypertrophy response of the thyroid to goitrogens being most effective in reducing thyrotropin production. Assays of pituitary thyrotropin content paralleled the thyroid function data. These experiments are interpreted as arguing against the existence of two distinct thyrotropins in the rat.

THE hypothesis that two distinct thyrotropic hormones may be elaborated by the rat hypophysis was first advanced by Greer (1) in 1952. The proposal was made in order to explain data which showed that anterior hypothalamic lesions eliminated the hypertrophic response of the rat thyroid to goitrogens (but not to exogenous thyrotropin (2)). These lesions did not decrease significantly the thyroid/serum radioiodide concentration ratio in the VanderLaans' "T/S test" (3) which Greer accepted as representative of "metabolic" response to thyrotropin stimulation. Greer's hypothesis was greatly strengthened by experiments in hypophysectomized mice bearing intraocular hypophyseal grafts (4), but the concept has generally proved unpopular. Several objections arose, centering about two facts which emerged subsequent to the publication of Greer's hypothesis. It was shown by Halmi *et al.* (5) that propylthiouracil (PTU), the goitrogen used by Greer to demonstrate a "goiter block" in appropriately lesioned animals, sensitized the thyroidal iodide trap to the action of thyrotropin (TSH). The Bogdanoves (6) have recently shown that the parallelism of response of the T/S ratio and thyroid weight to the level of circulating thyrotropin in rats receiving PTU was true only for the range examined originally by Greer. These findings permitted an alternate explanation of Greer's data in the rat, postulating a quantitative reduction in the level of a single TSH and a differential sensitivity of iodide trap and

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gland size to TSH. The work of VanderLaan and Caplan (7) also showed that factors other than thyrotropin could influence the activity of the iodide trap without necessarily affecting the gland size.

In a recent preliminary report by D'Angelo and Traum (8) a decrease in the thyroidal hormone release rate after hypothalamic lesions was demonstrated. In these animals the level of circulating TSH appeared to be reduced, although in a second series of lesioned animals the pituitary TSH stores appeared to be normal.

We undertook the present study in order to measure the effect of hypothalamic lesions on a number of criteria of thyroid function and to explore further the need for postulating two distinct thyrotropins in the rat.

MATERIALS AND METHODS

Adult male rats of the Holtzman strain weighing 300 to 450 gm. were lesioned as previously described by Greer (9), using a modified Krieg stereotaxic instrument. Bilateral lesions were placed 6 to 8 mm. anterior to the ear bars and 0.5 mm. to each side of the midline. The electrodes were inserted (with 0.5 mm. bared at the tip) to within 0.5 mm. of the base of the skull. A current of 2.5 milliamperes was applied for 15 seconds. After lesioning the animals were allowed to recover for at least two weeks or until their weight had returned to the pre-lesioning level.

The animals were then given a 12 μC dose of carrier-free I^{131}-iodide and the 24 hour uptake was determined with a collimated scintillation counter. Starting 72 hours after tracer administration, daily thyroid counts were performed under light ether anesthesia for ten days and the biological half-life of thyroidal radioiodine, corrected for physical decay, was determined graphically. A three ml. blood sample was then taken by cardiac puncture, and protein-bound stable iodine was determined on the serum by the method of Barker, Humphrey and Soley (10) and total cholesterol was assayed by the Abell method (11).

The animals then received a 15 μC dose of I^{131} and were placed 24 hours later on a diet to which 0.15% of PTU had been added. Two days later twice daily counting of the thyroid region was resumed. Twelve to 15 days after institution of the PTU regimen the T/S ratio was determined by the technique of VanderLaan and Greer (12).

We have arbitrarily defined as "goiter blocks" those lesions which limited the PTU—induced hypertrophy to 25% or less of the average weight of the thyroids of rats which succumbed to the lesioning procedure. Lesions which permitted a greater weight increase to occur were termed "ineffective," a designation which is meant to refer only to the effect upon thyroid weight.

Groups of about 20 animals (including controls) were studied. Animals which failed to survive the entire experimental sequence were excluded from the data here presented. Six groups, including a total of 56 lesioned and 43 control animals are described in this report.

At autopsy the brains of lesioned animals were fixed and then cut serially, and stained with cresyl violet acetate for histological localization of the lesions. Pituitaries were either stained with hematoxylin-eosin for eliminating the possibility of ischemic infarction, or else were kept in a deep freeze until assayed for TSH content by a modification of the iodine-discharge method of Gilliland and Strudwick which we have previously described (13). In experiment 6 thyroidal stable iodine was determined by the Barker method after dissolving the glands in 4 M sodium carbonate. In this experiment the rats did not receive PTU at any time.

TABLE 1. EFFECT OF LESIONS ON THYROID HYPERTROPHY AND FUNCTION

	Experiment 1		Experiment 2	
	Lesioned	Controls	Lesioned	Controls
No. rats	7	9	7	7
Weight (gm.)	403 ± 15*	313 ± 5	427 ± 11	378 ± 5
24-hr. I^{131} uptake (%)	4.4 ± 0.5†	11.2 ± 1.9	3.63 ± 0.20†	9.30 ± 0.34
Half-life of thyroid I^{131} (days)	4.2 ± 0.5	3.1 ± 0.3	8.1 ± 0.4†	4.7 ± 0.1
PBI (γ/100 cc.)	2.0 ± 0.2†	3.4 ± 0.1	4.7 ± 0.5	5.4 ± 0.2
Thyroid weight (mg.)	30.5 ± 1.4	32.4 ± 2.3	14.36 ± 0.55†	31.13 ± 1.44
T/S	178 ± 30	245 ± 26	390 ± 40†	179 ± 12
Serum cholesterol (mg. %)	—	—	111 ± 7†	72 ± 6
Adrenal weight (mg.)	35.5 ± 4.0	27.5 ± 2.0	31.8 ± 2.9	42.6 ± 1.3
Testicular weight (gm.)	2.72 ± 0.11	2.76 ± 0.06	3.16 ± 0.15	3.46 ± 0.11
Pituitary weight (mg.)	8.4 ± 0.3	9.5 ± 0.4	8.07 ± 0.36†	12.44 ± 0.38

* Standard error of the mean.
† Difference from control value significant at 1% level of confidence.

RESULTS

In Table 1 are summarized the results of Experiments 1 and 2. In experiment 1 lesions were placed 6 mm. anterior to the ear bars. This resulted in lesions caudal to the paraventricular nuclei but anterior to the median eminence as shown by histological study of the brains. Testicular and adrenal weights were normal. The pituitaries also were of normal size. In this series there was no marked effect upon the T/S ratio or thyroidal I^{131} half-life and the thyroids responded normally to PTU with hypertrophy. However, there were marked depressions of both the 24 hour I^{131} uptake and the serum PBI which were statistically significant.

In Experiment 2 lesions were placed 8 mm. anterior to the ear plugs in the area described as the "thyrotropin center" by Greer. These lesions were caudal to the supraoptic nuclei and destroyed the paraventricular nuclei. They did not affect the testicular weight, but did reduce adrenal and pituitary size without encroachment upon the stalk vessels or pituitary infarction. The PBI appeared to decrease slightly. No thyroid hypertrophy occurred in response to PTU. The 24-hour uptake was reduced to less than half of control values, the biological half-life of thyroidal I^{131} was greatly lengthened and serum cholesterol levels rose. The T/S ratio was twice as high as in the controls.

Table 2 summarizes experiments 3 to 5 in which lesions were placed 6.5 to 7.5 mm. anterior to the ear plugs. Only 12 out of the 35 animals lesioned developed "goiter blocks," although thyroid hypertrophy was significantly reduced (but not to goiter block levels) in the "ineffective lesions" of experiment 4. It is evident from tables 1 and 2 that lesions which do not block the goitrogen-induced thyroidal hypertrophy can affect thyroidal function severely. The reduction in the thyroidal function criteria is more pronounced in "goiter block" than in "ineffective" lesions, but uptake, hormonal discharge rate and PBI can all be reduced significantly by lesions

TABLE 2. EFFECT OF LESIONS ON THYROID HYPERTROPHY AND FUNCTION

Experiment	3			4			5		
Group	Goiter Block	Ineffective Lesion	Control	Goiter Block	Ineffective Lesion	Control	Goiter Block	Ineffective Lesion	Control
No. rats	3	7	5	4	11	11	5	10	7
Weight (gm.)	433±25*	455±9	449±17	384±14	388±13	408±7	415±30	427±14	362±21
24 hr. I^{131} uptake (%)	6.9±1.1†	9.3±1.0†	16.8±0.8	8.8±1.3	10.5±1.1	12.2±1.0	3.36±0.31†	5.39±0.64†	10.13±0.71
Half-life of I^{131} (days)	8.2±1.8	7.1±0.8	4.1±0.1	8.4±1.7	7.1±0.4	6.0±0.3	9.2±0.4†	6.7±0.2†	4.8±0.4
PBI (γ/100 cc.)	2.0±0.4	2.3±0.1	3.0±0.2	1.8±0.5	2.7±0.1†	3.3±0.1	2.8±0.2	3.2±0.2	4.1±0.3
Half-life on PTU (days)	2.8±0.6	2.7±0.4	2.0±0.1	4.2±1.2	2.7±0.2†	1.8±0.1	3.4±0.5	2.1±0.2	1.8±0.1
T/S	174±62	110±8	125±9	223±35†	85±11	88±13	199±48	191±23	138±25
Thyroid weight (mg.)	16.2±2.9	29.3±1.9	31.8±1.3	15.8±2.4†	27.9±1.2†	41.5±2.0	19.7±1.7†	33.0±1.5	38.2±2.2
Adrenal weight (mg.)	41.5±4.0	46.1±1.3	44.8±2.5	35.9±3.7	44.3±1.4	46.3±1.0	28.8±1.9	43.5±2.3	36.5±2.7
Testicular weight (gm.)	2.25±0.48	3.21±0.13	3.20±0.10	2.45±0.58	3.11±0.13	3.33±0.05	1.98±0.21	2.87±0.09	2.60±0.32
TSH per gland (mU.)	—	—	—	—	—	—	35	120	200
Serum cholesterol (mg.%)	—	—	—	—	—	—	115±8†	96±6	81±3

* Standard error of the mean.
† Difference from control value significant at 1% level of confidence.

TABLE 3. EFFECT OF LESIONS IN ANIMALS NOT TREATED WITH PTU

	Severely affected	Less affected	Controls
No. of rats	4	5	6
Weight (gm.)	460 ± 40*	416 ± 32	387 ± 10
24-hr. I^{131} uptake (%)	3.6 ± 0.3†	5.1 ± 1.1	8.2 ± 0.7
I^{131} Half-life (days)	8.7 ± 0.7	6.8 ± 0.3	7.2 ± 0.2
Thyroid I^{127} (γ/mg.)	1.64 ± 0.08	1.58 ± 0.20	1.93 ± 0.17
TSH (mu./gland)	74	210	400
Thyroid weight (mg.)	10.3 ± 0.8	15.0 ± 1.1	15.7 ± 1.8
Adrenal weight (mg.)	47.6 ± 3.4	46.5 ± 4.0	51.2 ± 3.5
Pituitary weight (mg.)	7.0 ± 1.0	8.6 ± 0.6	10.0 ± 0.5
Testicular weight (gm.)	0.92 ± 0.20†	2.0 ± 0.44	3.50 ± 0.12

* Standard error of the mean.
† Difference from control value significant at 1% level of confidence.

which do not prevent goiter formation. In Experiment 4, the T/S ratio was higher in the animals with goiter block lesions than in control animals. In Experiments 3 and 5 this was also true, but the differences from control values were not statistically significant at the 1% level. The animals having "ineffective" lesions had T/S values which were not significantly different from control values in any of these experiments.

Assays for hypophyseal TSH content after PTU feeding showed a decreased content per gland in "ineffective" lesions and a very marked decrease in the glands from "goiter block" animals.

In Table 3 are shown the results of Experiment 6 in which the use of PTU was avoided. It was therefore impossible to ascertain whether the lesions were truly "goiter blocking" or not. Using the results of the previous experiments, however, the animals were divided into a more- and a less-affected group as judged by iodine metabolism criteria. The thyroidal iodine stores were then assayed and found unchanged from control values. In these animals, as in those which had been treated with PTU, pituitary TSH stores were only 1/5 of normal in the animals showing the greatest changes in iodine metabolism, while the less affected animals had about 1/2 of the control TSH stores. In this experiment, the lesions were large and in many cases encroached upon centers known to influence gonadotropin secretion. However, we never found any correlation between testicular atrophy and the extent of reduction of thyroid function in the 11 animals in which atrophic testes were found at autopsy. Nor could the occurrence of hyperphagia or polydipsia be correlated with reduction of thyroid function. Gonad atrophy and hyperphagia were common in extensive lesions.

DISCUSSION

Our experiments show that lesions can have a range of effectiveness. The 24 hour uptake of I^{131} appears to be most sensitive to hypothalamic intervention, followed by the thyroidal radioiodine discharge rate and the PBI.

Of particular interest are the findings of Experiment 4 in which we found that the thyroid weight response can be significantly reduced by lesions which do not qualify as "goiter blocks" since the glands still hypertrophy strikingly, but do so less than the controls. The serum cholesterol level, which would be expected to be inversely proportional to thyroid function was highest in "goiter block" and less elevated in "ineffective" lesions. "Ineffective" lesions in the anterior hypothalamus appeared to cause smaller depressions of thyroid function than "goiter block" lesions, but the depression was quite clear nevertheless.

The "graded" response we observed would seem to support the thesis that hypothalamic lesions act by reducing pituitary TSH secretion. Our assay methods were not sufficiently sensitive to measure serum TSH levels, but D'Angelo and Traum (8) have shown that these are reduced by anterior hypothalamic lesions. Our assays of pituitary TSH content support their findings since we found that ineffective lesions caused a reduction to 60% of control levels and goiter block lesions reduced it to 20% in animals which had been administered PTU for two weeks. In Experiment 6, where no PTU was given, the TSH levels decreased similarly in the two lesioned groups when the assignment into the two groups was made on the basis of thyroid function. While it is not permissible to argue from pituitary stores to circulating serum levels, our data in conjunction with D'Angelo's findings would indicate that in lesioned animals TSH production is reduced. There does not appear to be any interference with the normal release mechanism and with the influence of PTU upon the release.

Our data concerning the total iodine contents of the thyroids of lesioned and control animals which had not received PTU indicate no changes which could be correlated with altered iodide uptake. This indicates that total glandular iodine content alone cannot explain the alterations in the iodide trap by the mechanism demonstrated by VanderLaan and Caplan (12). However, changes in the distribution of iodine among iodinated compounds in the gland do occur and are currently under investigation.

It is of some interest that pituitary size decreased in lesioned animals. In only one experiment was this decrease statistically significant at the 1% level, but it occurred also in the other two experiments in which pituitary weight was determined. This weight decrease was not due to ischemia or infarction. The decrease in pituitary weight may well be due to the decrease in the number of acidophil cells Bogdanove *et al.* (14) have described.

Our data would seem to be most readily interpreted by postulating that the production of a single thyrotropin is under partial hypothalamic control. A lower circulating TSH level would account for the response of all thyroid function aspects except the T/S ratio. It is not valid to consider differences in the T/S ratio between experimental animals showing great differences in thyroid size, and it is unfortunate that so much of the "two thyrotropin" hypothesis in the rat rests upon this criterion (1, 15). How-

ever, the present experiments need to be interpreted cautiously and probably apply to the rat only. There appear to be great differences in the control of thyroid function in various species. McMenamin, Florsheim and Sawyer (16) were unable to establish the existence of a thyrotropin-controlling area in the hypothalamus of the rabbit, and the work of Knigge and Solomon (17) in the hamster also indicates a hypothalamus-pituitary-thyroid interrelationship which differs from that found in the rat. A final conclusion of the controversy about the existence of more than one thyroid stimulating hormone will have to await further exploration of hypothalamus-pituitary-thyroid interrelationships in all of these species and a satisfactory explanation of the differences which have been reported.

SUMMARY

The response of thyroid function to anterior hypothalamic lesions has been determined in the rat using a number of thyroid function tests. The results of all tests excepting only the T/S ratio would appear to be most readily explained by an interference with the hypophyseal production of thyrotropin by such lesions, with "goiter block" lesions being most effective in reducing TSH production.

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THE EFFECT OF HYPOTHALAMIC LESIONS ON SEXUAL BEHAVIOR AND ESTROUS CYCLES IN THE EWE[1]

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ABSTRACT

The effect of hypothalamic lesions on estrous behavior, the pituitary content of gonadotrophin and the histologic picture of the ovary, the uterus, the adrenals, and thyroid have been investigated by the stereotaxic production of electrolytic lesions of the hypothalamus in twelve cycling ewes. Destruction limited to the massa intermedia or optic chiasm (3 animals) had no effect on cycles, but lesions in the ventral hypothalamus with a common area just above the median eminence anteriorly abolished heat (9 animals). There were no changes in the adrenals, and only slight changes in the thyroids of the animals with lesions. Two of the animals in which heat was abolished showed large corpora lutea at autopsy, and another one of these animals had a mature follicle. On the other hand, in one of two animals in which cycles were abolished a low pituitary content of both FSH and LH was found. In the other, a reduced pituitary content of LH was demonstrated. Both showed histologically anestrous uteri and small pituitaries. The pituitary gonadotrophin content of all of the remaining lesioned animals without cycles was not essentially different from that of animals in which cycles continued. It is concluded that lesions in the anterior hypothalamus abolish heat in the estrous ewe without, at least in some cases, changing the cyclical endocrine relationship between the pituitary and ovary.

IN ANIMALS like the rabbit, which ovulate only after copulation, and in spontaneously ovulating polyestrous species like the rat, there is considerable evidence that sexual cycles are at least in part under hypothalamic control. This evidence has been discussed in detail in a review by Harris (1). Gonadal function in the ferret, an animal which is anestrous in the winter and estrous in the summer, is also probably under diencephalic control (2). There are, however, no data on the role of the hypothalamus in the control of estrous cycles in sheep, a species in which polyestrus alternates with anestrus. Accordingly, the following studies were undertaken to

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nvestigate the effect of hypothalamic lesions on estrous behavior and the pattern of gonadotrophin secretion in the ewe.

MATERIALS AND METHODS

Twelve sexually mature, cycling ewes, of mixed breed, were studied. All animals were maintained in pens and fed hay and concentrates. They were checked daily for receptivity to the male by placing them with a vasectomized or aproned ram.

After at least one and in many instances two or three heats were observed, electrolytic lesions were placed stereotaxically in the hypothalamus of each of the twelve animals by a modification of the technique of Hume and Ganong (3). With the animal's head fixed

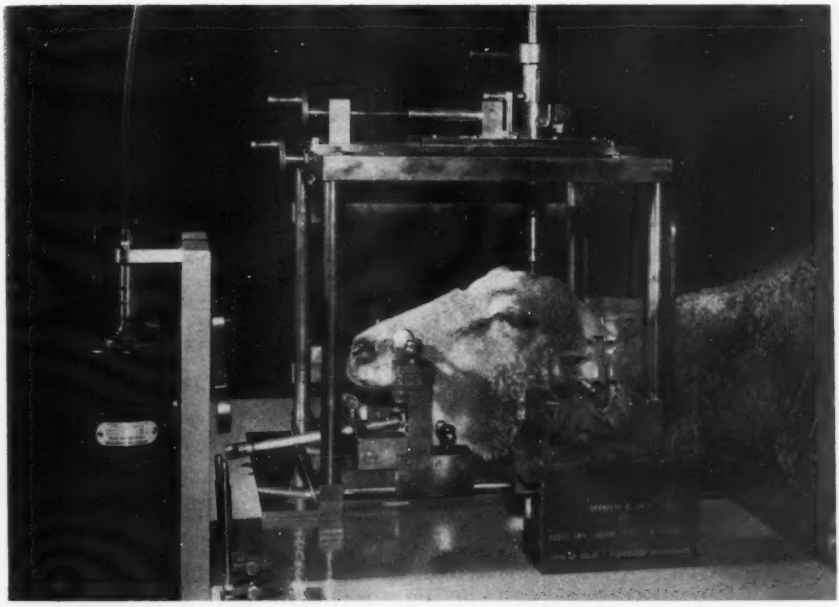

FIG. 1. Photograph of stereotaxic apparatus with sheep's head in position.

in the stereotaxic machine (Fig. 1), a hole was drilled in the skull, and a twenty-six gauge stainless steel needle inserted into the third ventricle. An injection of 0.15 cc. of a 70% solution of acetotriazoate was made and a lateral X-ray film taken. The ventriculogram thus obtained outlined the mammillary bodies, the optic chiasm, and the massa intermedia (Fig. 2). Simple adjustments of the stereotaxic apparatus permitted the insertion of electrodes with a high degree of accuracy into any desired portion of the brain bounding the third ventricle. In most instances, two electrodes were used, and a direct current of 6 milliamperes for two to three minutes applied to each. Postoperatively the animals did well and daily checks for heat continued for 60 days, or in three instances, until the animals were sacrificed.

At autopsy, the hypothalamus was removed, fixed in 10% neutral formalin and serially sectioned transversely at 15 micra. Each twentieth section was stained with hematoxylin and eosin and examined. Study of these sections permitted the reconstruction of the lesion in each individual animal on a diagram of the mid-sagittal section of the

hypothalamus and pituitary. The thyroid and adrenals were dissected and weighed. These tissues, as well as specimens of the uteri were fixed in Bouin's solution for subsequent staining with hematoxylin and eosin and histologic examination. The number and size of follicles and number of corpora lutea in each ovary were recorded at the time of autopsy and the reproductive tract was examined grossly.

The whole pituitary was removed as rapidly as possible after death, weighed, frozen in dry ice and the posterior lobe removed. The anterior lobes were lyophilized and the dried powder assayed for FSH and LH. FSH was determined by a modification of the method of Steelman and Pohley (4). A mixed suspension of 16 mg. of anterior lobe powder and 10 I.U. of human chorionic gonadotrophin (HCG) was injected subcutaneously twice daily for three days into 22-day old Sprague-Dawley female rats. LH was

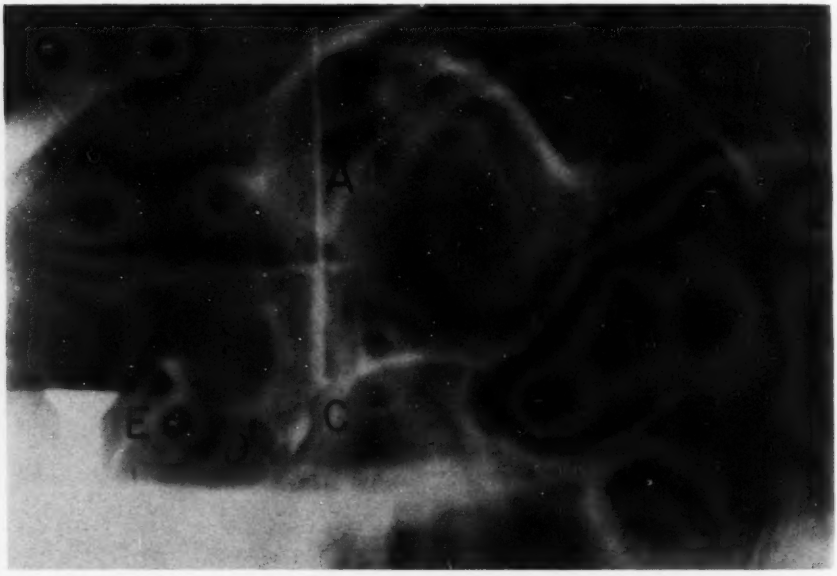

FIG. 2. Ventriculogram of sheep's brain. (A) Stainless steel needle inserted into the third ventricle; (B) Massa intermedia; (C) Optic chiasm; (D) Mammillary body; (E) Aqueduct of Sylvius.

assayed on the basis of the ventral prostate response of 22-day old hypophysectomized male Sprague-Dawley rats injected intraperitoneally twice daily for four days with a total of 2 mg. of a suspension of the dried anterior lobe (5). The results for each pituitary were quantitated by comparison with standard FSH and LH preparations.[3]

In Northern California, this mixed breed of sheep begins to show recurrent periods of heat about the first of August and, in most cases, becomes anestrous by the following March or April. In the present experiments, checking for cycles was begun in October, lesions were produced in October and November, and except for three animals which were sacrificed at the end of November, the ewes were killed the following March.

[3] Follicle stimulating hormone (FSH), Lot No. R377201 and Luteinizing hormone (LH), Lot No. R3772424 were generously provided by Dr. Irby Bunding of Armour Laboratories.

TABLE 1. EFFECT OF HYPOTHALAMIC LESIONS ON THE ESTROUS CYCLE IN SHEEP

Ewe number	Lesion location	Dates of heat before lesion	Date of lesion	Dates of heat after lesion	Date of autopsy	Ovarian histology		Uterine histology
						Diameter of largest follicle (mm.)	No. corpora lutea present	
5621	Massa intermedia	11–1, 11–16	11–30	12–18, 1–4 1–19	3–22	4	0	cyclic
5504	Massa intermedia	11–10	12–4	1–7	3–20	5	0	cyclic
2523	Optic chiasm	9–7, 9–24	10–8	10–13, 10–30, 11–17	11–23	5	2	cyclic
5881	Over stalk	11–25	12–4	none	3–23	3	1	cyclic
5872	In stalk and above	11–29	11–30	none	3–23	4	2	cyclic
3369	Anterior median eminence	9–11	10–8	none	11–23	10	0	cyclic
5480	Posterior tuber, over stalk	11–15	12–4	none	3–21	19*	0	cyclic
5960	Anterior median eminence	10–25, 11–11	11–27	none	3–23	5	0	cyclic
3595	Over stalk	8–27, 9–12, 9–28	10–8	none	11–23	3	0	acyclic
5825	Stalk and above	10–21, 11–4, 11–20	11–27	none	3–22	4	0	acyclic
5440	Stalk, posterior tuber	11–10, 11–26	11–30	none	3–20	4	0	cyclic
5987	Posterior tuber	11–19	12–4	none	3–23	5	0	cyclic

* Degenerating.

RESULTS

The effects of the lesions on estrous behavior and the histology of the ovaries and uterus are summarized in Table 1. Six of the animals had one heat before lesions were produced, four had two, and two had three heat periods. Postoperatively, in three animals, heat continued to occur recurrently. One of these animals had only one observed cycle before being killed, but the other two had three each at regular 17-day intervals. Lesions in two of these animals were in the massa intermedia, and in the other, the optic chiasm.

The remaining nine animals had no postoperative heat periods. Lesions in these animals involved the ventral hypothalamus bilaterally, in and near the median eminence. They ranged from the back of the optic chiasm anteriorly to the anterior edge of the mammillary bodies posteriorly, but all lesions had in common a small bilateral area of destruction located just above the anterior third of the median eminence (Fig. 3).

One of the three animals in which cycles continued after lesion production was killed in November seven days after a heat period. This animal had two normal appearing corpora lutea. The remaining two animals were killed in March. The ovaries of both animals showed only small to medium size follicles and no corpora lutea. Among the animals with no cycles postoperatively, six had only small to medium follicles in their ovaries. However, two animals in this group, three months after their last observed behavioral heat, had well developed corpora lutea in their ovaries, and one animal had a large mature follicle. It is of some interest that all three of these animals had lesions anterior to the pituitary stalk.

One non-cycling animal (ewe 5480) had a very large abnormal follicle. The histological picture showed a very thin thecal layer and degenerating granulosa cells. Because of this anomaly, the ovarian weight of this ewe

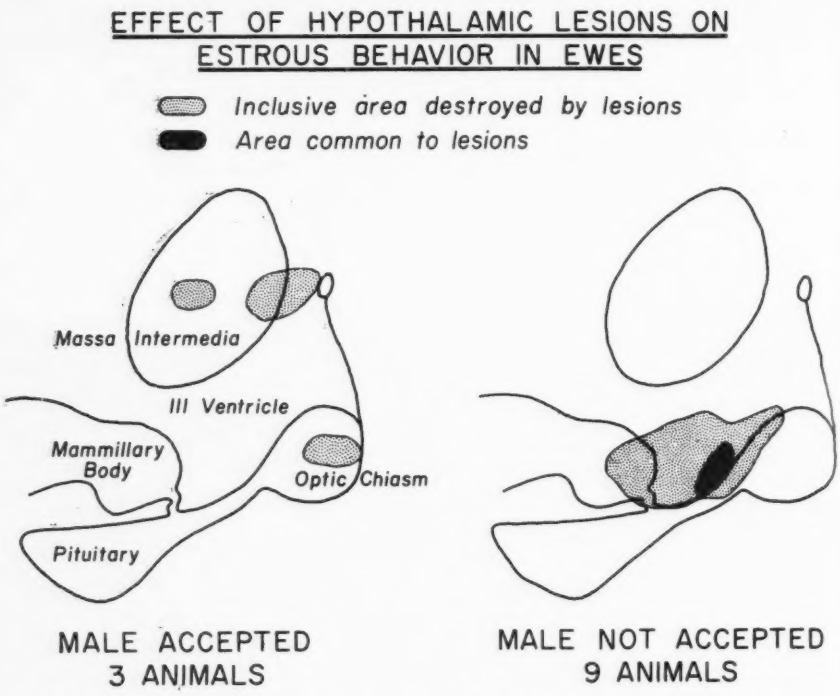

FIG. 3. Diagram illustrating the areas in which lesions were placed. The lesions are projected on a mid-sagittal section of the hypothalamus.

was greater than normal. In one other non-cycling animal (ewe 5825), the ovarian weight was smaller than normal. With these two exceptions, the size of the ovaries was not different from normal sheep.

On the basis of the microscopic appearance of the uterine epithelium and the size of the uterine glands, as well as the amount of edema or extravasation in the uterine mucosa, the uteri were classified as either cyclic or acyclic. No further attempt was made to differentiate these tissues with respect to stage of the estrous cycle. The uteri of the animals in which regular heat periods continued were judged to be cyclic. In two of the nine animals in which estrous behavior was inhibited (ewe 5825 and ewe 3595) the histological picture of the uterus was that of anestrus, but in the remaining seven a cyclic pattern was present.

The assays of the pituitary for gonadotrophic activity are summarized in Table 2. Ewe 5825 had a low content of both LH and FSH, but the pituitary was small in this animal and the lesion involved much of the pituitary stalk. Accordingly, infarction of the anterior lobe may well have been present (6). The remaining animals in which heat periods were abolished did not show pituitary FSH and LH levels significantly different from those in the animals which continued to cycle. Pituitaries of normal non-operated

TABLE 2. PITUITARY GONADOTROPHIN CONTENT OF SHEEP WITH HYPOTHALAMIC LESIONS

	Material injected	Average ovarian wt., mg. (mean ± S.E.)	Average ventral prostate wt., mg. (mean ± S.E.)	FSH A.U. per ant. lobe	LH mg. equiv. per ant. lobe	Total dried ant. lobe wt., mg.
	Anterior lobe from donor ewe number					
Ewes refusing male	5825	(2) 51.1 ± 13.6	(4) 14.3 ± 1.3	0.41	1.5	48
	5960	(6) 48.9 ± 3.9	(5) 21.2 ± 3.4	0.85	7.6	108
	5440	(6) 63.9 ± 8.0	(6) 20.5 ± 3.1	2.45	10.9	157
	5872	(5) 70.6 ± 5.4	(5) 19.6 ± 1.0	1.16	9.6	99
	5480	(6) 45.4 ± 2.3	(3) 17.0 ± 3.4	0.81	9.0	120
	5987	(6) 59.5 ± 5.8	(1) 23.6	1.28	24.2	149
	5881	(6) 73.1 ± 5.6	(6) 18.9 ± 1.1	2.05	15.4	164
	3369	(5) 51.1 ± 3.7	(5) 30.0 ± 1.8	1.13	58.5	180
	3595	(4) 87.3 ± 6.7	(6) 19.4 ± 1.3	1.38	7.2	80
Ewes accepting male	2523	(5) 52.8 ± 5.3	(5) 36.8 ± 3.0	1.56	85.0	200
	5504	(5) 64.3 ± 4.1	(5) 24.8 ± 3.5	1.58	23.6	126
	5621	(6) 51.5 ± 4.1	(5) 17.9 ± 2.1	1.27	7.5	141
	Standard FSH					
	0.1 A.U.	(6) 52.0 ± 5.8				
	0.5 A.U.	(12) 110.7 ± 8.2				
	1.0 A.U.	(12) 134.7 ± 9.7				
	Standard LH					
	0.105 mg.		(9) 15.5 ± 1.2			
	0.212 mg.		(8) 24.0 ± 2.0			
	0.848 mg.		(4) 38.4 ± 9.5			
Control		(8) 35.3 ± 1.3	(13) 8.4 ± 0.47			

S.E. = standard error; A.U. = Armour unit.
Numbers in parentheses represent the number of rats used per assay group.

animals were not assayed in the present study.

The histological picture and weight of the adrenal glands in all animals were essentially the same as those found in normal cycling sheep (7). No abnormalities of the cortex or medulla were seen. The height of the thyroid epithelium was lower than normal in four animals. Three of these ewes had large stalk lesions. The differences, however, between these and the other animals with lesions were slight, and further data on the function of the thyroid in these animals would be necessary before definite conclusions could be drawn.

DISCUSSION

In the present study, heat was clearly inhibited by lesions in the ventral hypothalamus. Whether the effects were limited to the behavioral manifestations of the estrous cycle or included interruption of the endocrine relationship between the pituitary and ovary is more difficult to decide. The available data which can be brought to bear on this problem are (1)

the ovarian changes found at autopsy, (2) the changes in the uterus, (3) and the pituitary content of gonadotrophic hormones.

There are apparently no data available on the picture of the ovary in the hypophysectomized sheep. However, in the immature and mid-anestrous animal, the ovary contains no corpora lutea and shows only small to medium follicles, the largest diameter usually reported being about 5 mm. During the polyestrous part of the year, the ovarian picture will include, of course, large, mature follicles and corpora lutea. The corpus luteum in the ewe reaches its maximum size at six to eight days after estrus, begins to regress at about the fourteenth day, and at the end of the second cycle is a mere vestige (8). Therefore, the presence of corpora lutea or large mature follicles indicates stimulation of the ovary by the pituitary. Six of the animals with lesions in which behavioral heat was abolished had only small to medium follicles in their ovaries, but two had fresh corpora lutea and one had a large mature follicle. In the latter three animals, therefore, there is presumptive evidence that only the behavioral manifestations of estrus were inhibited.

Similarly, all but two of the animals in which cycles were abolished showed a uterine histological picture which was not anestrous. These results also suggest that there was no deficiency of pituitary gonadotrophin secretion in most of the non-cycling animals.

The final evidence which can be brought to bear on this problem is the data on gonadotrophin content of the pituitary glands. It is admittedly difficult to generalize from the pituitary content of a given hormone with respect to its rate of secretion into the blood stream. In the rat, however, it has been claimed that gonadotrophin assays of the pituitary are a very sensitive indicator of the gonadotrophin deficiency induced by hypothalamic lesions (9, 10). These experiments indicated that even when the gonads were of normal size, there was often marked depression of the gonadotrophin content of the pituitary glands. In the present study, all animals but one among the ewes in which heat was abolished showed gonadotrophin levels which were not significantly different from those found in the ewes in which cycling continued. The one animal with a low content of both hormones, as noted above, had a large stalk lesion, and probably some pituitary infarction. Recent studies have shown that extensive necrosis of the anterior lobe follows stalk section in the sheep (6).

Santolucito (7) has studied the gonadotrophin content of the ewe pituitary at various stages of the estrous cycle, using the same assay methods employed in the present study. The FSH values he observed were not significantly different from the values in the present study with the exception of ewe 5825. On the other hand, the LH values he reported were generally higher than those found in our animals with hypothalamic lesions. The extent to which his values can be compared to ours is limited, however, since he did not study anestrous animals and he did not use comparable standard

pituitary gonadotrophic hormone preparations.

The conclusion seems justified, on the basis of the cyclic histological picture of the uterus and the absence of marked depressions of pituitary FSH and LH content, that pituitary secretion of these gonadotrophic hormones continued in at least some of the animals with hypothalamic lesions in the absence of behavioral heat. Whether or not this secretion was sufficient to bring about normal periodic ovulation cannot be determined from this study. To answer this question a repetitive visualization of the ovary in the live animal is required. These studies are underway at the present time.

Absence of mating behavior following hypothalamic lesions has been reported in a variety of species. The early experiments of Dempsey and Rioch (11) and Bard (12) suggested the existence of a center in the posterior hypothalamus or rostral mesencephalon which controlled estrous behavior in the female guinea pig and cat, but subsequent studies have shown that ventral anterior hypothalamic lesions block mating in both species (13, 14, 15, 16). Some evidence suggests the existence of a similar center in the rat (17, 18). Furthermore, both Brookhart and his collaborators (13), and Sawyer and Robinson (16) have shown that this effect can be produced without interruption of pituitary gonadotrophic activity. The present studies confirm these observations and add the ewe to the list of animals in which ventral hypothalamic lesions just behind the optic chiasm abolish heat without apparently altering pituitary-ovarian endocrine activity.

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LIPID-SHIFTING PROPERTIES OF 17α-METHYL-17-HYDROXY-5(10)-ESTREN-3-ONE,[1] A STEROID WITH BOTH ESTROGENIC AND ANDROGENIC ACTIVITIES

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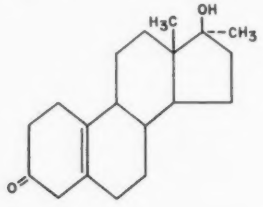
ABSTRACT

17α-Methyl-17-hydroxy-5(10)-estren-3-one (SC-6582) possessed approximately 11 to 40% the activity of estrone in depressing the plasma cholesterol-phospholipid ratio in cholesterol-fed cockerels, whereas, it had only 0.1 to 0.9% the activity of estrone when tested on uterine growth of mice, and only about 0.2% when evaluated with rat vaginal smears. The reduction in cholesterol-phospholipid ratio for both estrone and the compound was due to an increase in plasma phospholipids with no reduction in plasma cholesterol. In addition to lipid-shifting and estrogenic activities, the compound was a weak androgenic and myotrophic agent. The possible effects of a compound possessing such a mixture of activities on the blood lipids of man have been discussed.

THE predominance of coronary artery disease in males over premenopausal females and the blood lipid-shifting activity of estrogens have resulted in the exploration of the possible utility of estrogens in the treatment of atherosclerosis (1). Although the inhibition and reversal of cholesterol-induced coronary atherosclerosis in chickens is well known (2), definitive evidence that estrogens are beneficial in the treatment of coronary disease in man is still lacking. The chronic administration of estrogens in male patients has been hampered by the feminizing properties of these agents. It was considered desirable, therefore, to search for a substance that would promote beneficial shifts in blood lipids and yet be largely devoid of estrogenic properties. This report summarizes biological studies on 17α-methyl-17-hydroxy-5(10)-estren-3-one (SC-6582), a steroid possessing potent lipid-shifting properties with relatively weak estrogenic and androgenic activities. This compound has the following structure:



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[1] This compound was synthesized by Dr. Frank B. Colton of the Division of Chemical Research, G. D. Searle & Co. U.V. absorption spectroscopy offered a sensitive and quantitative measure of possible contamination by aromatic or conjugated steroids. By means of such studies the maximum aromatic estrogen or conjugated androgen contaminations were 0.3 and 0.02% respectively. These amounts of impurities could not account for the biological properties reported in this paper.

Methods. Lipid studies were carried out in Hy-line cockerels 6½ weeks old at the beginning of the experiments. During test they were fed a mash containing 2% cholesterol and 5% cottonseed oil. All test substances were dissolved in corn oil and administered subcutaneously; control birds were given corn oil in volumes equivalent to those given treated birds. Injections were given daily for 3 days (except for the 15-day tests), and 24 hours following the last dose, blood samples were taken from the wing vein in heparinized syringes. Plasma total cholesterol was determined by the method described by Zlatkis, *et al.* (3). The lipid phosphorus, determined by Sperry's method (4), was multiplied by the factor 25 to give the amount of total phospholipids.

Estrogenic activity was determined from uterine growth studies in mice and vaginal smear studies in rats. For mice the assay procedure of Rubin, *et al.*, as modified by Edgren (5), was followed. Vaginal smear studies were conducted according to the method described by Edgren and Calhoun (6).

Androgenic and myotrophic activities were determined in immature male rats by the method of Eisenberg and Gordan (7). The compound was administered in 7 equal doses, starting 3 weeks after castration. The rats averaged 150 gm. at the start of the injection period and were sacrificed on the day after the last injection. Gains in body weight, as well as terminal weights of the seminal vesicles, ventral prostate glands and levator ani muscles, were examined and compared with these parameters in controls autopsied at the same time.

Results. Lipid Effects. The plasma cholesterol-phospholipid ratio increased from approximately 0.6 to 1.8 in untreated cholesterol-fed control birds (Fig. 1). This increase resulted from a rise in plasma cholesterol with little or no change in the phospholipid level. In birds treated with SC-6582 or estrone, the plasma cholesterol and phospholipid were increased above controls; however, the effect on phospholipids was much greater than on cholesterol, resulting in a decreased C/P ratio (Table 1). The decreases in C/P ratio were linearly related to log-dose for both SC-6582 and estrone (Fig. 1). Since the slopes of the dose-response curves were significantly different, no single relative potency value could be assigned. Compared to an arbitrary low dose of estrone (1.0 mg./kg.), SC-6582 had a relative potency of 40% and at a high dose of estrone (5.0 mg./kg.) SC-6582 had 11% the potency of estrone.

Both SC-6582 and estrone were similar in their effects on body weight but were strikingly dissimilar in their effects on comb growth (Table 1). SC-6582 significantly stimulated whereas estrone inhibited comb growth. The fact that this comb growth stimulation with simultaneous depression of the C/P ratio was evident during continuous administration of SC-6582 for a longer period of time is illustrated in Figure 2.

Estrogenic Activity. Since the slopes of the dose-response curves for SC-6582 and estrone were significantly different, no single relative potency

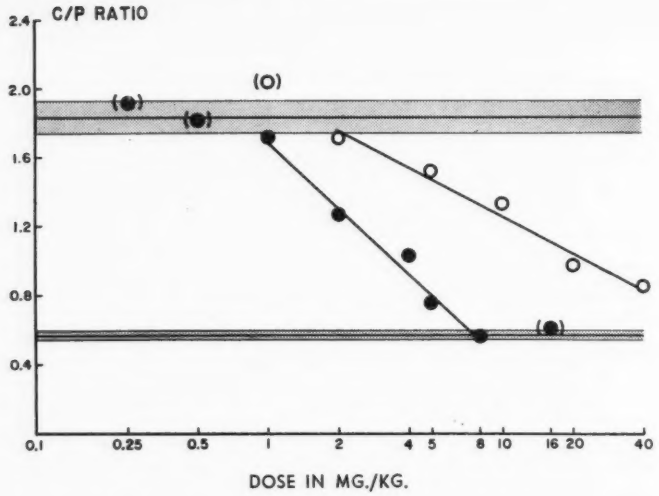

FIG. 1. Effect of SC-6582 and estrone on reduction of the plasma cholesterol-phospholipid ratio in cholesterol-fed cockerels. Closed circles = estrone; open circles = SC-6582. Each point represents the mean of 6 to 28 animals. Regression lines fitted to points not in parentheses by method of least squares. Lower and upper shaded areas indicate mean and 95% confidence limits for concurrent controls before and after treatment respectively.

value could be assigned (Fig. 3). An arbitrary low dose of estrone in this test was 0.1 μg. which was equivalent to 11.0 μg. of SC-6582, indicating that the compound had approximately 0.9% the activity of estrone. At the highest dose level employed, SC-6582 was about 0.1% as estrogenic as estrone.

SC-6582 was also tested for estrogenic activity in a vaginal smear assay in rats. Using a modification of Horn's (8) method, the ED_{50} of SC-6582

TABLE 1. EFFECTS OF SC-6582 AND ESTRONE ON PLASMA LIPIDS, BODY WEIGHT AND COMB GROWTH IN CHOLESTEROL-FED COCKERELS

Treatment		No. of chicks	Plasma lipids			Body weight gain, gm.	Comb growth length + height, mm.
Compound	Dose, mg./kg.		Cholesterol, mg.%	Phospholipid, mg.%	C/P		
Pooled Controls		62	445 ± 14*	246 ± 5.4	1.83 ± .05	48 ± 3	+4.7 ±
SC-6582	1.	8	553 ± 25	272 ± 11	2.04 ± .07	50 ± 10	+7.4 ±
	2.	12	651 ± 43	379 ± 20	1.71 ± .06	41 ± 5	+5.4 ±
	5.	12	736 ± 60	498 ± 53	1.52 ± .09	61 ± 6	+5.8 ±
	10.	20	890 ± 40	684 ± 38	1.33 ± .05	69 ± 7	+6.5 ±
	20.	11	975 ± 51	1005 ± 47	.98 ± .06	68 ± 5	+9.0 ±
	40.	6	1277 ± 92	1540 ± 178	.86 ± .09	63 ± 12	+16.0 ± 1
Estrone	.25	16	592 ± 54	331 ± 42	1.92 .13	55 ± 5	+1.7 ±
	.5	12	551 ± 31	312 ± 21	1.82 ± .11	43 ± 6	.0 ±
	1.	16	582 ± 27	345 ± 20	1.72 ± .06	48 ± 5	−2.5 ±
	2.	28	826 ± 45	698 ± 36	1.27 ± .06	70 ± 5	−3.7 ±
	4.	19	973 ± 57	954 ± 61	1.03 ± .04	71 ± 5	−3.0 ±
	5.	16	1315 ± 64	1735 ± 79	.77 ± .03	118 ± 6	−3.4 ±
	8.	16	1165 ± 56	2077 ± 84	.57 ± .02	77 ± 7	−2.5 ±
	16.	12	1270 ± 84	2113 ± 139	.62 ± .04	82 ± 14	−3.0 ±

* Mean ± S.E.

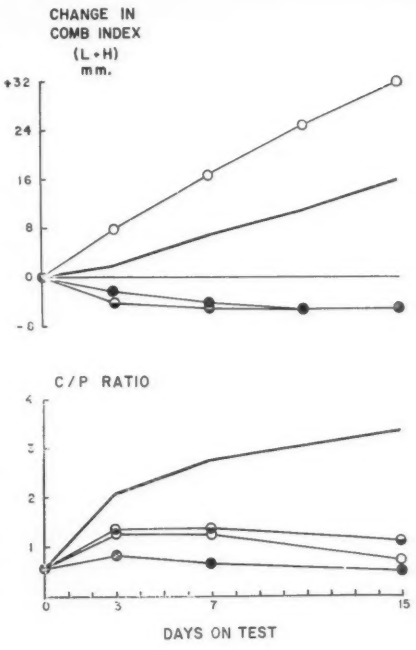

FIG. 2. Effect of SC-6582 and estrone on comb growth and reduction of the plasma cholesterol-phospholipid ratio in cholesterol-fed cockerels. Closed circles = estrone 5 mg./kg.; half-filled circles = estrone 2 mg./kg.; open circles = SC-6582 10 mg./kg.; heavy solid line = controls. Each group contained 8 birds and doses were given daily subcutaneously in oil.

was estimated as 1600 μg., while that for estrone was 2.38 μg. Thus, SC-6582 had about 0.2% the activity of estrone in this test.

Oral Activity. Studies for estrogenic activity in the mouse showed that SC-6582 was active following intragastric administration. The slope of the dose-response curve was parallel to that obtained following subcutaneous administration (Fig. 3). The oral-parenteral ratio was about 0.4.

Androgenic and Myotrophic Activities. SC-6582 possessed androgenic activity as shown by the response of the seminal vesicles and ventral prostate and myotrophic activity as shown by the levator ani response (Table

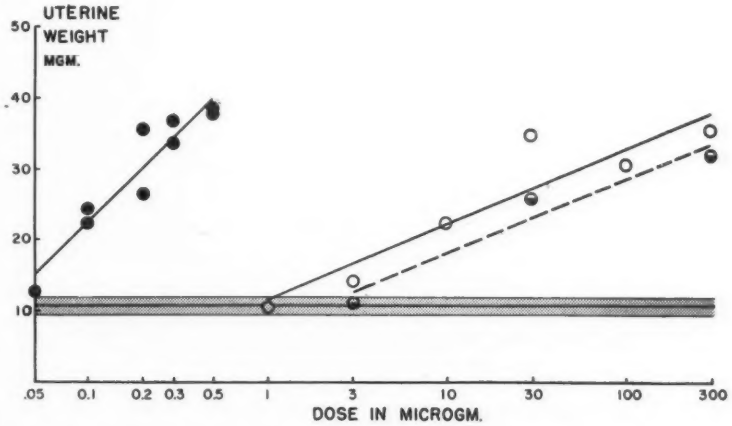

FIG. 3. Effect of SC-6582 and estrone on uterine weight in immature female mice. Closed circles = estrone; open circles = SC-6582 administered subcutaneously; half-filled circles and broken line = SC-6582 administered intragastrically. Each point represents the mean of 8 to 10 animals. Regression lines fitted by method of least squares. Shaded area indicates control mean and 95% confidence limits.

TABLE 2. EFFECTS OF SC-6582 AND TESTOSTERONE PROPIONATE ON BODY WEIGHT, SEMINAL VESICLES, VENTRAL PROSTATE AND LEVATOR ANI IN RATS, 3 WEEKS POST-CASTRATION

Treatment		Body weight gain, gm.	Seminal vesicles, mg.	Ventral prostate, mg.	Levator ani, mg.
Compound	Total dose, mg.				
Controls	—	37.0±1.3*	7.5± .4	9.8± .8	60.5±2.1
SC-6582	1.	23.1±2.2	11.9± .4	16.4± .9	67.3±4.1
	2.	16.4±2.1	17.4±1.4	15.1±1.1	88.0±5.5
	5.	9.7±2.2	29.3±1.6	21.0±1.8	106.4±4.1
Testosterone propionate	.05	34.1±1.8	11.2± .8	15.0±1.1	61.2±5.3
	.2	33.4±1.3	43.0±3.4	47.2±3.2	87.3±4.3

* Mean ±S.E., groups of 8 rats were used at each dose level.

2). The compound was about one-twentieth as androgenic as testosterone propionate at low dose levels but the dose-response curve had a lower slope. The myotrophic effect was about one-tenth that of testosterone propionate. It was noteworthy that SC-6582 showed the myotrophic response despite a simultaneous catabolic effect on body weight, which is a typical estrogenic property.

Discussion. These data suggested that the lipid-shifting and estrogenic properties of a molecule may be separable. 17α-Methyl-17-hydroxy-5(10)-estren-3-one (SC-6582) possessed approximately 11 to 40% of the activity of estrone in depressing the cholesterol-phospholipid ratio in cholesterol-fed cockerels, whereas it had only 0.1 to 0.9% the activity of estrone when tested on uterine growth of mice, and only about 0.2% when evaluated with rat vaginal smears. In addition to lipid-shifting and estrogenic activities, SC-6582 was a weak androgenic and myotrophic agent; potentially it may be hoped that this androgenic-myotrophic activity would offset the feminizing properties. The simultaneous depression of C/P ratio and stimulation of comb growth certainly supported the hypothesis of separability and may indicate an inhibition of feminization. Stamler, *et al.* (9) have reported that the combined administration of estrogen and androgen will prevent estrogen-induced feminization without interfering with the estrogen-induced prophylactic inhibition of cholesterol-induced chick coronary atherogenesis. This inhibition was associated with a depression of the C/P ratio except when the androgen dosage exceeded a certain level. Preliminary studies with combinations of estrogen and androgen in man suggested that androgens may inhibit the estrogenic effects on blood lipids often without reversing feminization (1). In contrast, androgens appear to have little effect on blood lipids of cockerels. Thus it would be difficult to predict the clinical activity of SC-6582 which incorporates lipid-shifting, estrogenic and androgenic activities in a single molecule. Although the

androgenicity of the compound may inhibit feminizing effects in human subjects, such activity may also be sufficient to block estrogenic effects on blood lipids.

Acknowledgment

The authors gratefully acknowledge the assistance of Mr. David W. Calhoun in the statistical calculations used in this study.

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INFUSION OF ALDOSTERONE, 9-α-FLUOROHYDROCORTISONE AND ANTIDIURETIC HORMONE INTO THE RENAL ARTERY OF NORMAL AND ADRENALECTOMIZED, UNANESTHETIZED DOGS: EFFECT ON ELECTROLYTE AND WATER EXCRETION[1]

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ABSTRACT

The development of a technique for chronic catheterization of one renal artery, with a collection of urine from each kidney individually, has afforded the opportunity of studying the effects on water and electrolyte excretion produced by aldosterone, 9-α-fluorohydrocortisone and ADH infused directly in the renal artery of normal and adrenalectomized dogs. In the normal dog intrarenal aldosterone or 9-α-fluorohydrocortisone produced a unilateral kaluresis but no demonstrable natriuresis, or effect on water excretion. In the same catheterized dogs, following recovery from adrenalectomy, small doses of aldosterone infused into the renal artery produced a unilateral anti-natriuresis. The unilateral kaluresis was of the same magnitude as that observed in the normal state. In both the normal and adrenalectomized animal the effect of intrarenal aldosterone on potassium excretion was apparent only after a delay of 30 to 60 minutes, and a maximum effect was noted after two to three hours. The present investigation also clearly indicates the local renal action of ADH on water excretion and the rapidity of its action. No natriuresis was observed with ADH in these normal dogs on a water diuresis.

THE mineralocorticoids have long been known to play an important role in the maintenance of extracellular sodium, but their site of action is still controversial. Loeb *et al.* (1) contended that the adrenal steroids controlled extracellular sodium by regulating the renal excretion of sodium, a concept long challenged by Swingle (2), and more recently by Overman (3) and Wilson (4). Similar differences of opinion exist concerning the mechanism of the elevation of serum potassium following adrenalectomy. Furthermore, although aldosterone has been shown to be a potent salt-retaining hormone, a direct demonstration of the renal tubular effect of the hormone has not been reported. Nor have such experiments been described for the direct renal effect of the antidiuretic hormone of the pituitary gland. The recent development of a technique for chronic catheteriza-

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[3] Student Research Fellow.

ion of one renal artery, with collection of urine from each kidney individually (5), has afforded the opportunity of studying the direct renal effects of aldosterone, 9-α-fluorohydrocortisone, and antidiuretic hormone on sodium, potassium and water excretion in the normal and adrenalectomized, unanesthetized dog.

METHODS

Four trained, female, mongrel dogs weighing 17–22 kg. were kept on a constant diet throughout these studies. Operative procedures were performed aseptically under sodium pentobarbital anesthesia. The left renal artery was catheterized according to a technique previously described (5). The polyvinyl catheter was flushed each day with concentrated heparin solution (10,000 units per ml.) and closed with a stainless steel obturator. The urinary bladder was hemisected sagittally between the ureteral orifices and two bladder pouches constructed. Urine from each kidney was then collected from lucite funnels implanted in the bladder pouches. After studies were completed in the normal dogs, bilateral adrenalectomy was performed transabdominally in two of the catheterized dogs. These adrenalectomized animals were maintained on 25 mg. per day of cortisone acetate by mouth, and in addition, desoxycorticosterone (1 mg. per day i.m.) as needed to maintain normal serum sodium and potassium levels. The last intramuscular injection of desoxycorticosterone was given four days before each experiment and the last cortisone was administered three days before each experiment.

Saline Diuresis. The dogs were fasted for 20 hours prior to each experiment but allowed water ad libitum. The unanesthetized animals were then placed in a Pavlov stand and given 500 ml. of isotonic saline (sodium concentration approximately 145 meq. per liter) intravenously over a 20-minute period. A constant intravenous infusion of the same solution was then continued at the rate of 4 ml. per minute for the duration of the entire experiment. When the urinary excretion of sodium, potassium and water reached a relatively constant level, control periods were started. After two or more 20–30-minute control periods the renal artery infusion was started and continued for 100–120 minutes at the rate of 0.25 ml. per minute. The intravenous infusion was continued during the intrarenal artery injection and for one or more periods after the end of the intrarenal artery infusion. The steroids were dissolved in absolute ethyl alcohol and then diluted with 5% dextrose in water. Similar amounts of alcohol and dextrose solutions used in control experiments had no effect on sodium, potassium or water excretion. When renal clearances were performed creatinine and sodium *p*-aminohippurate were added to the priming solutions and to the constant intravenous infusion.

Water Diuresis. The dogs were fasted for 20 hours and allowed water ad libitum. The animals were given water by stomach tube until a brisk water diuresis was started. They were then placed in a Pavlov stand and the diuresis maintained by hourly loading with 250 ml. of water. When the water excretion was relatively steady antidiuretic hormone (pitressin-vasopressin injection) diluted in 5% dextrose and water was infused into the renal artery at the rate of 0.25 ml. per minute.

Analyses. Sodium and potassium determinations in serum and urine were made with a flame photometer using an internal standard. Creatinine and *p*-aminohippurate were determined by standard procedures.

OBSERVATIONS

A. *Intrarenal infusion of aldosterone and 9-α-fluorohydrocortisone in normal dogs.* The difficulty of demonstrating the antinatriuretic effect of the mineralocorticoids in acute experiments in the normal dog is well known.

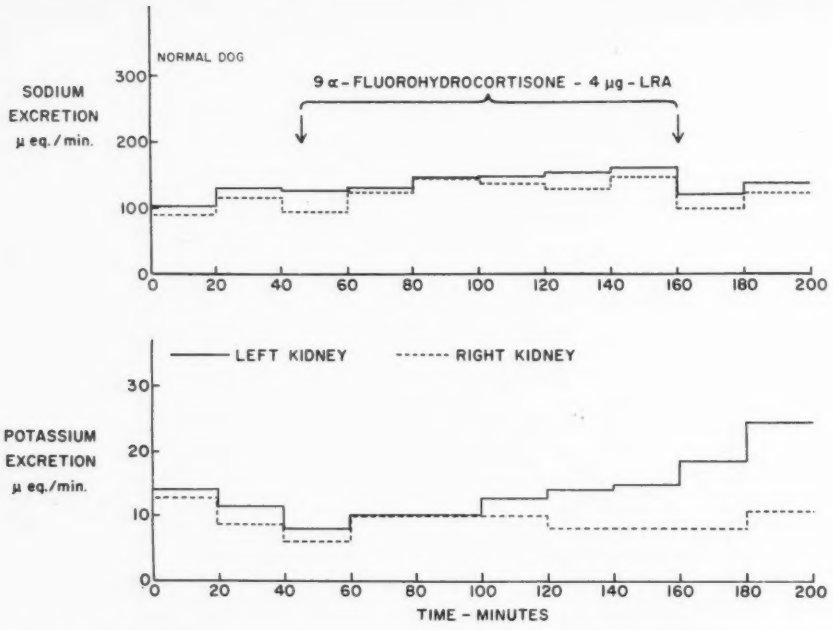

FIG. 1. A representative example showing the effect of left intrarenal artery infusion (LRA) of 9-α-fluorohydrocortisone (4 μg.) on sodium and potassium excretion in the normal unanesthetized dog.

Moreover, as indicated in Figures 1 and 2 the same problem is encountered even with direct intrarenal artery infusion of 9-α-fluorohydrocortisone and aldosterone in doses which produce a profound antinatriuresis in the adrenalectomized dog (Fig. 4). With the normal animal maintained on a constant intravenous infusion of isotonic saline and excreting 100–150 μeq. of sodium a minute from each kidney the intrarenal infusion of 4 μg. of 9-α-fluorohydrocortisone over a 2-hour period produced no effect on sodium excretion on the infused side (Fig. 1). However, a unilateral kaluresis was observed on the infused side, with potassium excretion on the left increasing in stepwise fashion above that on the right, which remained relatively constant. The intrarenal infusion of 10 μg. of aldosterone similarly had no effect on the rate of sodium excretion but did have a definite effect on potassium excretion (Fig. 2A). In this latter experiment sufficient aldosterone spilled over into the systemic circulation to produce a kaluresis on the right side as well, which was less than that observed on the infused or left side in any given period. It is of interest that the potassium excretion from the right kidney in the period 150–180 minutes, for example, was equal to the excretion from the left kidney during period 120–150 minutes, and, in general, the excretion on the right side was similar to that on the

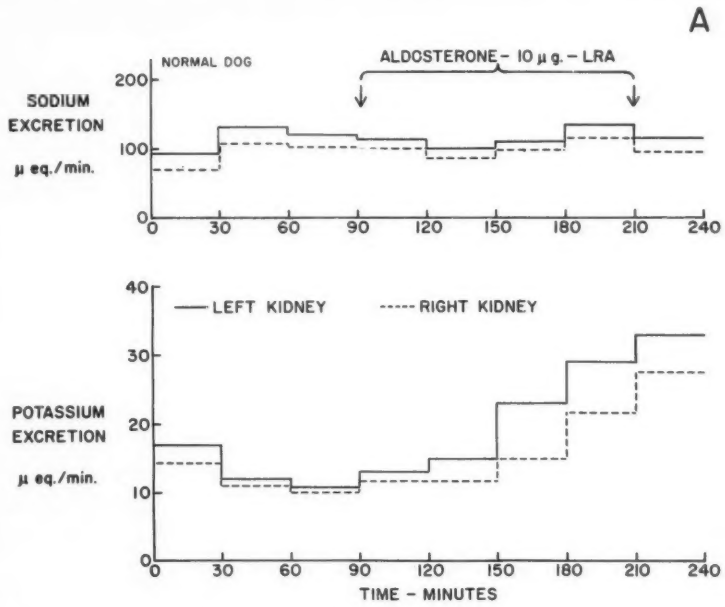

FIG. 2a. A representative example showing the effect of left intrarenal artery infusion (LRA) of aldosterone (10 μg.) on sodium and potassium excretion in the normal unanesthetized dog.

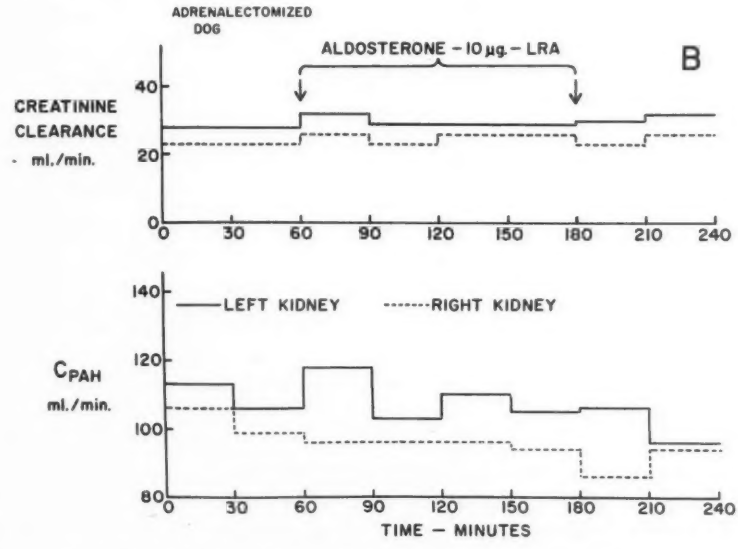

FIG. 2b. Creatinine and *p*-aminohippurate clearances in the same experiment illustrated in 2a.

left side in the previous period. The kaluretic effect was observed in each experiment with 9-α-fluorohydrocortisone or aldosterone infused intrarenally in the normal dogs. There was no significant effect of either of these steroids on sodium excretion, water excretion, or on creatinine or para-aminohippurate clearances (Fig. 2B).

B. *Intrarenal infusion of aldosterone and 9-α-fluorohydrocortisone in adrenalectomized dogs.* Since we were unable to demonstrate the antinatriuretic effect of aldosterone or of 9-α-fluorohydrocortisone by intrarenal infusion in the normal dog, two dogs that had been used in the normal state studies were adrenalectomized. Desoxycorticosterone and cortisone therapy were withdrawn four and three days respectively prior to the experiment. Figure 3A illustrates results obtained by the infusion of 0.45 μg. of aldosterone into the left renal artery of the adrenalectomized animal. Prior to the intrarenal infusion of aldosterone the sodium excretion on the left side was uniformly higher than that on the right, and this was consistently the case in control studies in this dog. During the intrarenal infusion the relationship between the excretion of sodium on the left and on the right was reversed with the left falling below that on the right. This altered relationship persisted for at least 2 hours after the end of the infusion, but in the overnight specimen the sodium excretion on the left was again higher than that on the right. The unilateral kaluretic effect was also present and quantitatively similar to that observed in the same dog before adrenalectomy. In order to compare quantitatively the magnitude of the effect of aldosterone on sodium and potassium excretion from the infused kidney, the differences between the left and right kidney in sodium and potassium excretions are plotted on the same scale in Figure 3B. The cumulative decrease in sodium excretion on the left side in this and other experiments was 2 to 3 times as large as the cumulative increase in potassium excretion. With an intrarenal infusion of 1 μg. of aldosterone over one hour there was evidence of slight spillover into the systemic circulation. However, at the peak of action of the steroid the sodium excretion on the infused side had fallen 42 μeq. per minute behind that on the right (average of control periods—left renal excretion of sodium exceeded that on the right by 18 μeq.) while the potassium excretion on the left exceeded that on the right by 27 μeq. per minute (average of control periods—left and right equal).

The latency of action of the mineralocorticoids is more clearly seen in the onset of increase of potassium excretion (Figs. 1A and 3), since its random variation is less than that of the sodium excretion. A definite increase in potassium excretion is not observed for approximately 60 minutes from the start of the steroid infusion in both normal and adrenalectomized animals with low rates of infusion. The maximum fall in sodium excretion and the maximum rise in potassium excretion occurred between 2 and 3 hours from the beginning of the infusion.

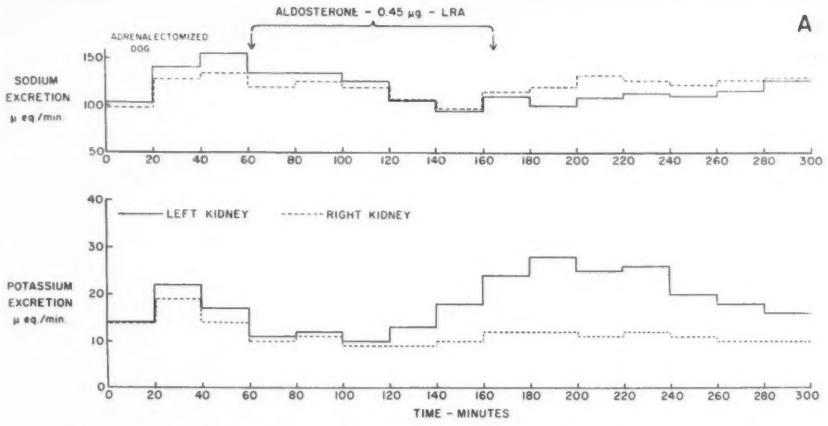

FIG. 3a. Effect of left intrarenal artery infusion of aldosterone (0.45 μg.) on sodium and potassium excretion in the adrenalectomized animal.

Figure 4A illustrates another experiment in the adrenalectomized dog in which the dosage of aldosterone was increased from 0.45 μg. (Fig. 3A) to 10 μg. infused over the same period of time, that is, increasing the rate of infusion from 0.0045 μg. per minute to 0.8 μg. per minute. The onset of the kaluresis is delayed for approximately 30 minutes with this larger dose suggesting that the latency may be altered by the size of the dose. The magnitude of the observed kaluresis was approximately the same as that observed prior to adrenalectomy with the same dosage (compare Fig. 2A and Fig. 4A). There is seen now, however, a profound antinatriuresis, slightly

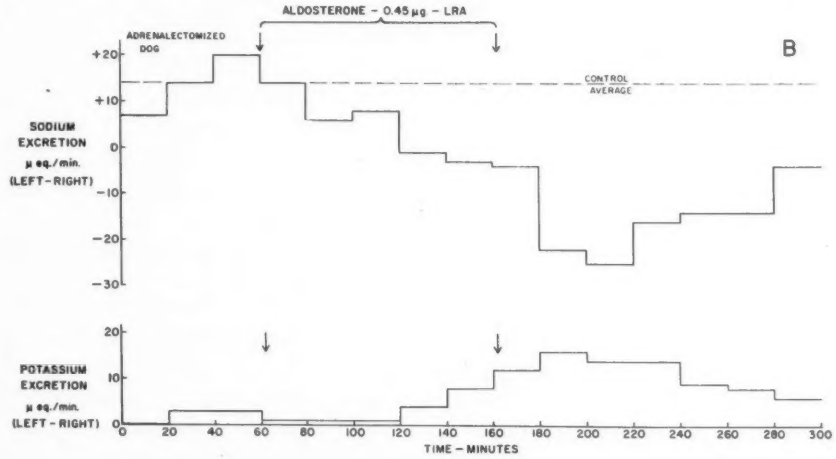
ALDOSTERONE - 0.45 μg. - LRA
ADRENALECTOMIZED DOG
B
CONTROL AVERAGE
SODIUM EXCRETION μ eq./min. (LEFT - RIGHT)
POTASSIUM EXCRETION μ eq./min. (LEFT - RIGHT)
TIME - MINUTES

FIG. 3b. Differences between the left and right kidney in sodium and potassium excretion produced by unilateral infusion of aldosterone. Data from Fig. 3a.

more pronounced on the infused or left side. This is in marked contrast to the observations made in the same dog with intact adrenals and the same dosage of aldosterone and with approximately the same control sodium excretion rates. Figure 4B indicates that the changes in sodium excretion were unaccompanied by alterations in creatinine and para-aminohippurate

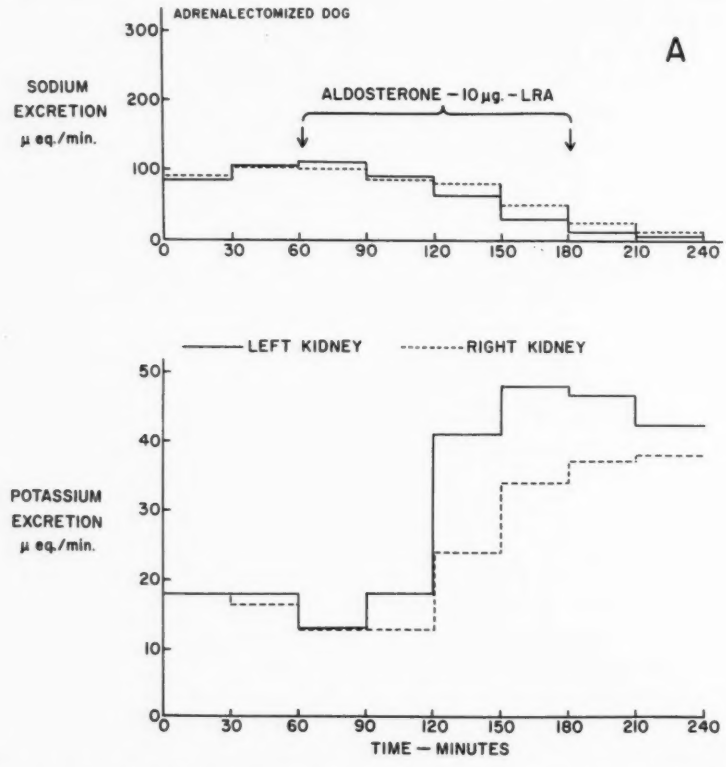

FIG. 4a. Effect of left intrarenal artery infusion of aldosterone (10 μg.) on sodium and potassium excretion in adrenalectomized dog.

clearances. The effects of administration of the same amount (10 μg.) of aldosterone intravenously may be observed in Figure 5. Excretion patterns on both right and left sides were almost identical indicating that the catheterized kidney responded in exactly the same fashion as the control kidney to an intravenous infusion of aldosterone.

C. *Intrarenal infusion of antidiuretic hormone.* The effects of intrarenal infusion of antidiuretic hormone on sodium and water excretion were studied in four normal dogs. Figure 6 is a representative example of the results obtained in 7 experiments. The infusion of 0.8 milliunits of anti-

diuretic hormone into the left renal artery over a 4-minute period produced a fall in urine flow from the infused kidney from the control rate of 1.5 ml. per minute to 0.9 ml. (a 40% fall) with no change in urine flow from the right or control kidney. The infusion of 1.6 millinunits of ADH into the left renal artery over an 8-minute period (that is, at the same rate as in the first infusion) produced a fall in urine flow from 1.5 ml. per minute to 0.55

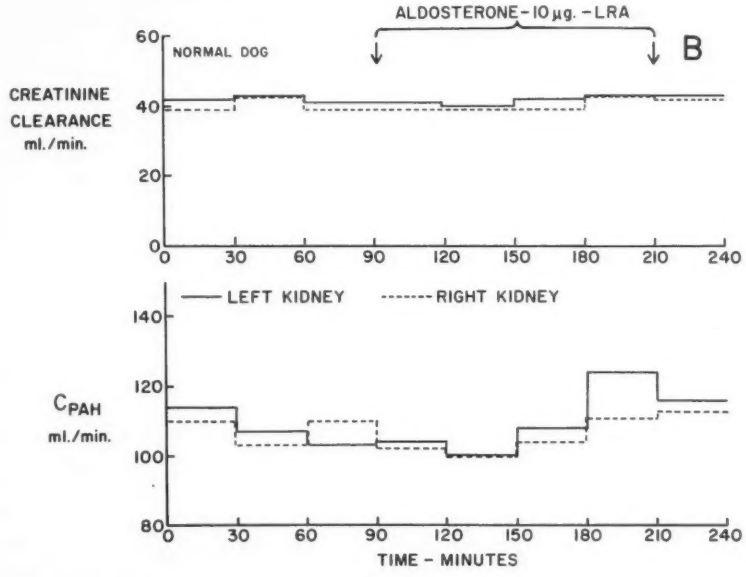

FIG. 4b. Creatinine and *p*-aminohippurate clearances in the experiment illustrated in Fig. 4a.

ml. per minute (a 60% fall) on the injected side. A small antidiuresis, lasting about 30 minutes, was noted in the control kidney after the second infusion, suggesting that sufficient hormone spilled over into the systemic circulation to produce a slight effect on the opposite kidney. In each infusion of antidiuretic hormone into the renal artery the decrease in water excretion was apparent in the first 10-minute collection period, that is, in the same period during which the hormone was given. The infusion of 4 milliunits of ADH intravenously in the same animal produced a 60% fall in total urine flow with equal effects on the two kidneys. From other experiments with intrarenal infusion of ADH it appears that the minimum dose which produced a detectable antidiuresis in one kidney was approximately 0.2 milliunits (Table 1). In the experiment illustrated (Fig. 6) no significant change in sodium excretion was observed. Over the range of antidiuretic hormone infusions employed (0.2 to 1.6 milliunits) a unilateral

TABLE 1. ADH INFUSION INTO THE LEFT RENAL ARTERY

Dog No.	Dose	Change in urine flow of left kidney (ml./min.)	% decrease
1	0.2 mU.	1.8 →1.4	22
1	0.3	2.0 →1.5	25
1	0.8	2.0 →1.2	40
2	0.8	1.75→0.75	57
3	0.8	2.75→1.95	30
4	0.8	1.5 →0.9	40
4	1.6	1.55→0.55	60

rise of sodium excretion was observed only once in 7 experiments in 4 dogs on water diuresis.

DISCUSSION

These studies were undertaken to determine the renal effect of aldosterone, 9-α-fluorohydrocortisone and antidiuretic hormone infused directly into the renal artery of the unanesthetized dog. The present investigation indicates that in the normal animal on an isotonic saline diuresis, and ex-

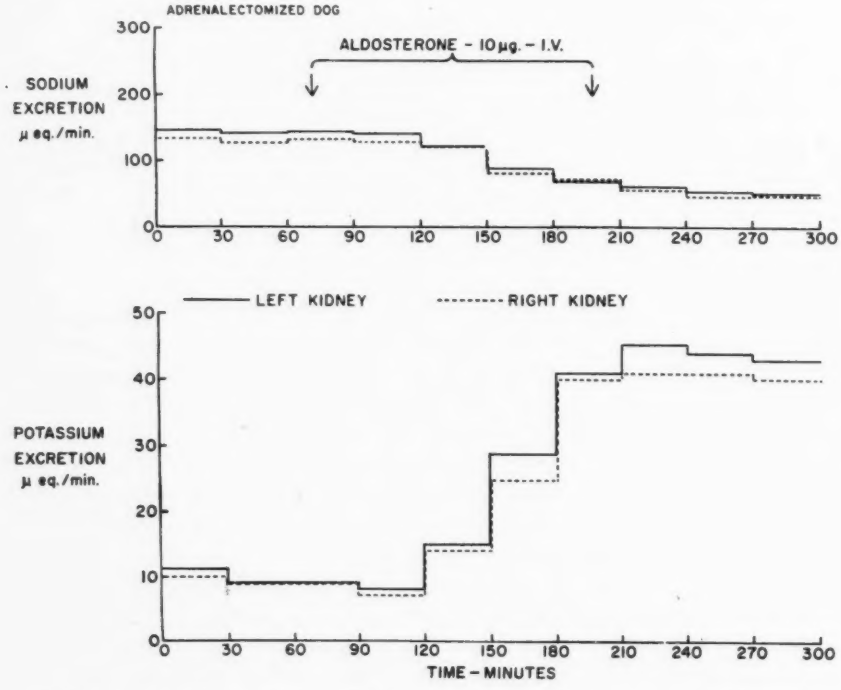

FIG. 5. Effect of intravenous infusion of 10 µg. of aldosterone on sodium and potassium excretion in the same dog illustrated in Fig. 4.

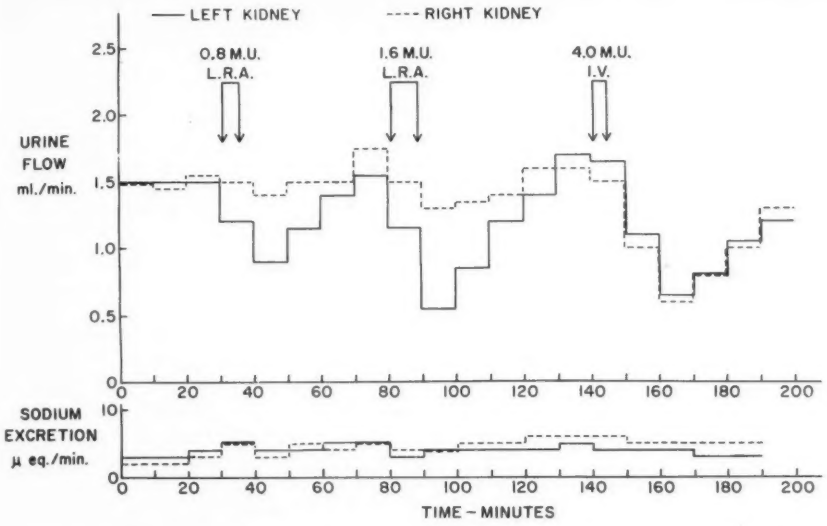

FIG. 6. A representative example showing the effect of left intrarenal artery infusion (LRA) of antidiuretic hormone on urine flow and sodium excretion in the normal unanesthetized dog. The effect is compared with that observed with an intravenous dose of antidiuretic hormone in the same animal.

creting 100–200 μeq. per minute of sodium from each kidney, the intrarenal infusion of the two mineralocorticoids over a wide dosage range did not produce a detectable decrease in sodium excretion from the injected kidney. In contrast, both steroids, at all rates of infusion employed, produced a marked kaluresis. This kaluresis was limited to the infused side when the hormones were injected at a slow rate (Fig. 1), thus indicating a direct renal effect of the steroids on potassium excretion, but no detectable effect on sodium excretion in the normal animal. Our data do not distinguish whether the kaluresis is a result of increased secretion of potassium or decreased absorption of potassium. Renal clearances of creatinine and paraminohippurate were unchanged.

In both of the catheterized dogs, after adrenalectomy, a direct or unilateral renal antinatriuretic effect of the steroids as well as a kaluretic effect could be demonstrated when the animals had been untreated for several days prior to the experiment. In these experiments in adrenalectomized animals the control rates of sodium and potassium excretion were nearly identical with those observed in the experiments on the normal animal (100–200 μeq. of sodium per minute per kidney and 10–15 μeq. of potassium per minute per kidney). Quantitatively, the effects on potassium excretion were similar in the normal and adrenalectomized dog. Thus, the steroids produce an effect on potassium excretion which is similar in both the normal and adrenalectomized animal, and yet produce no effect on

sodium excretion in the normal state. Studies are now being made to determine whether or not there is a direct renal antagonism between aldosterone and other hormones secreted from the adrenal cortex. Nevertheless, when an effect of the steroids on sodium excretion was observed, i.e., in the adrenalectomized state, the magnitude of the antinatriuresis was two to three times greater than the kaluresis, emphasizing the differing response of the normal and adrenalectomized animal. Apparently, only with some deviation from the normal state is the unilateral or renal effect on sodium excretion observed. This decrease in sodium excretion occurred without change in glomerular filtration rate, demonstrating the increased tubular reabsorption of sodium produced by the steroids in the adrenalectomized animal. However, the magnitude of the unilateral change observed with 0.45 to 1.0 μg. of aldosterone infused into the left renal artery compared with that observed with the intravenous infusion of 10 μg. of the hormone still does not enable us to answer the question whether the total decrease in sodium excretion seen with a larger dose given intravenously can be explained entirely on the direct renal effect of the hormone. Furthermore, the fall in sodium excretion from each kidney produced by the intravenous infusion of 10 μg. of aldosterone does not differ substantially from the fall observed with the intrarenal infusion of the same amount of aldosterone.

The separation of the action of aldosterone on sodium and potassium excretion in the normal dog, and the two to threefold greater effect on sodium excretion as compared to potassium excretion in the adrenalectomized dog, do not support the hypothesis of a simple cation exchange of sodium and potassium in the distal tubule, but rather to an independence of movement. Similar independence of movement of sodium and potassium has been observed in kidney slices (6) and in yeast and mitochondria (7). Foulkes *et al.* (8) have recently shown *in vivo* the selective action of protamine on potassium transport in the kidney without effect on sodium transport. These observations on the direct renal effect of the steroids on potassium and the lack of effect on sodium in the normal animal may explain the kaluresis seen in primary hyperaldosteronism, and the absence of edema formation.

A direct and rapid unilateral renal effect of the antidiuretic hormone on water excretion was clearly seen in the normal dog. However, an effect on sodium excretion was demonstrated only once in the seven experiments in four dogs on water diuresis. Whether a change in sodium excretion might be produced by the intrarenal infusion of antidiuretic hormone under other experimental conditions (e.g. isotonic saline diuresis) is yet to be determined.

Acknowledgment

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METABOLISM OF CORTISOL-4-C^{14} IN THE DOG HEART-LUNG PREPARATION[1,2]

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ABSTRACT

The metabolism of cortisol-4-C^{14} has been studied at physiologic concentrations in two dog heart-lung preparations. The first preparation survived for 100 min. and the second for 75 min. An average of 68% of the radioactivity added was recovered from the heart-lungs. An average recovery of 79% was obtained from blood specimens to which cortisol-4-C^{14} was added to produce a concentration similar to that in specimens from the heart-lungs.

No evidence of metabolism of cortisol was seen in control blood specimens incubated 100 min. at room temperature or at 37° C. Approximately 10% of the radioactivity added to the heart-lung preparations was recovered in a fraction which appears to be 11β, 17α, 20β, 21-tetrahydroxy-Δ^4-pregnene-3-one. The remainder of the recovered radioactivity appears to be unaltered cortisol-4-C^{14}.

LIVER has the ability to degrade cortisol rapidly as demonstrated by *in vitro* studies with acellular preparations (1, 2, 3, 4), liver slices (5), liver perfusions (6, 7, 8), and by *in situ* experiments (9).

However, there have been conflicting reports concerning the existence of extra-hepatic systems for altering cortisol. Nelson *et al.* (10) found no arteriovenous difference in plasma phenylhydrazine chromogens across inflamed human limbs, even when corticosteroid levels were elevated by ACTH infusion. Wilson *et al.* (11) reported that inflamed human knee joints are capable of converting cortisol to a variety of products. Ganis *et al.* (12) isolated and identified four steroid metabolites, including 11β, 17α, 20β, 21 tetrahydroxy Δ^4 pregnene-3-one, from bovine kidney minces incubated with cortisol. Lombardo *et al.* (13) found a substance more polar than cortisol in extracts of cortisol-perfused surviving dog kidneys. This substance was not demonstrated to be a steroid. Robbins *et al.* (8) found "no appreciable change" in phenylhydrazine chromogen of blood perfused one hour through the hind limbs of a rat. Sweat *et al.* (14) have reported the isolation and identification of six different labeled steroids from fibroblast-derived tissue culture incubated with cortisol-4-C^{14}.

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The data of the present report indicate that the dog heart-lung preparation metabolizes cortisol at an exceedingly slow rate compared to liver and that a product of this metabolism appears to be 11β, 17α, 20β, 21 tetrahydroxy Δ^4 pregnene-3-one.

METHODS

Two heart-lungs were prepared from approximately 10 kg. dogs by a technic modified after that described by Knowlton and Starling (15). In this preparation the left ventricular output is directed through an adjustable resistance and a warming coil to a reservoir connected to the superior vena cava. All other effluent and affluent channels are ligated. Thus a closed circuit including only heart, lungs, and connecting vessels is achieved. The blood donor and the experimental dogs were male mongrels. The animals were anesthetized with sodium pentobarbital (30 mgm. per kg.), given intravenously. Blood clotting was prevented by the intravenous administration of 30 mgm. of heparin sodium.

Cortisol-4-C^{14}, specific activity 1.47 millicuries per millimole, was obtained from the Endocrinology Study Section of the National Institutes of Health. Two mg. of this material were chromatographed in a propylene glycol:toluene system for 5 days. Eighty-two per cent of the radioactivity was found in a two inch zone. The steroid in this area was eluted and used in the experiments described below.

Following completion of all manipulations required in the preparation of the heart-lung, 235 μg. of cortisol-4-C^{14} in 3 ml. of ethanol were added dropwise to the venous reservoir. To evaluate recovery and possible metabolism of steroid by blood, 80 μg. of cortisol-4-C^{14} were added to each of two 400 ml. aliquots of donor dog blood with agitation. One aliquot was incubated in a water bath (38° C) for a period coincident with the survival of the heart-lung and the other was allowed to stand at room temperature (non-incubated control). Both aliquots were swirled occasionally during the experiment.

The experiment was terminated when a mean arterial pressure of 100 mm. Hg could no longer be maintained, despite adjustment of peripheral resistance and reservoir level. Deterioration of the preparation was associated with progressive pulmonary edema and cyanosis. The duration of the experimental period in dog 1 was 100 min. and in dog 2, 75 min.

At the end of the experiment the blood was drained from the preparation. The heart and lungs were dissected from the mediastinum. All specimens, including whole blood, were stored frozen until extraction. Prior to extraction, the heart and lungs were ground separately to a thick slurry in small quantities of ethanol with a Waring blendor.

Blood and tissue specimens were extracted with 2.5 volumes of absolute ethanol by shaking in glass-stoppered centrifuge bottles. After centrifugation and removal of the supernatant the residues were twice extracted with a volume of 70% ethanol equal to 1.5 times that of the original tissue slurry or blood. Ethanol extracts were combined. The residues from the ethanol extractions were twice extracted with a volume of chloroform equal to 2.5 times that of the tissue slurries or blood. For each tissue the combined chloroform and ethanol extracts were mixed in a large separatory funnel. The mixtures separated into aqueous and chloroform-ethanol phases. Emulsions were broken by standing two hours at −20° C. The aqueous layer was washed three times with an equal volume of chloroform.

The chloroform-ethanol phase was dried at reduced pressure under a stream of nitrogen at a temperature under 40° C. The dried residue was redissolved in 10 ml. of 70% ethanol per 25 gm. of original tissue or blood. The ethanol extracts were defatted by shaking with 10 ml. of n-hexane per ml. of 70% ethanol. The hexane was decanted and the procedure repeated with fresh hexane, 5.0 ml. per ml. of 70% ethanol. The ethanol was drawn off and diluted to a volume of approximately one ml. per gram of original

tissue or blood. These experiments contained 1 to 3 mg. of non-volatile substance per ml.

Aqueous, hexane, and ethanolic fractions were plated and counted under a thin window gas-flow tube (Nuclear-Chicago D-47). Counts were made in duplicate to a 10% standard error and corrected by means of an experimentally determined self-absorption curve.

Isolation of steroids was accomplished by descending chromatography according to principles described by Burton, Zaffaroni, and Keutmann (16). For preliminary resolution and quantitation of the steroids present, aliquots of ethanol extracts equivalent to 25 gm. of blood or tissue were chromatographed 5 to 8 days on strips 3 cm. wide in a propylene glycol:toluene system. Larger amounts of steroid were required for identification studies and were isolated in a formamide:chloroform system in which development requires only 6 to 8 hours. Chromatographic patterns were identical in the two systems. Acetylated derivatives were chromatographed in a propylene glycol:toluene system, 17-ketosteroid derivatives in a propylene glycol:n-hexane system.

Acetates were prepared by dissolving evaporated, vacuum dessicated residues in one ml. of a 2:1 pyridine:acetic anhydride. The mixture stood for 20 hours at room temperature. The acetylating mixture was evaporated under a stream of nitrogen *in vacuo* at room temperature. 17-Ketosteroid derivatives were prepared as described by Berliner *et al.* (17). Phenylhydrazine chromogens were determined by incubating a 2 ml. methanol solution of the steroid with 3 ml. of a 0.15% (w/v) solution of phenylhydrazine hydrochloride in 22N sulfuric acid overnight in darkness at room temperature. Extinction was determined at 420 millimicrons.

Radioactivity was located on paper chromatograms with a recording scanner (Nuclear-Chicago, Actigraph C-100). This instrument provides a chart record of the radioactivity on the paper chromatogram.

Identification studies were based on the mixed radiochromatogram technic. The radioactive zones on the chromatograms revealed as peaks on the record of the scanner were separately eluted in methanol. The amounts of radioactive steroid in these eluates ranged from 1 to 20 micrograms, quantities too small for physical and chemical characterization by conventional means.

The mobility of a radioactive unknown provides evidence for a reasonable conjecture regarding its identity. On the basis of this conjecture, 50 to 75 micrograms of an appropriate authentic steroid sample were added to the labelled unknown. The mixture was chromatographed and the resulting paper scanned. The strip was cut into three-fourths inch sections, each of which was eluted in 4 ml. of methanol. The optical density at 240 mμ. of each eluate was determined. These values were plotted on the scanner record of the radioactivity. A precisely similar distribution of radioactivity and ultraviolet light absorbing power indicated tentative identity of the labelled unknown and the added known specimen.

Additional evidence of identity was obtained by performing the same procedure after acetylation and after oxidation. In the case of cortisol the distribution of phenylhydrazine chromogenicity was also determined before and after acetylation.

RESULTS

Table 1 shows the recovery of activity from each specimen, expressed as a percentage of the amount added. Virtually all of the radioactivity was found in the ethanol fraction. The hexane and aqueous fractions each contained amounts of the order of 0.1% of the total. The absence of significant radioactivity from the aqueous fraction is regarded as evidence against conjugate formation.

TABLE 1. RECOVERY OF RADIOACTIVITY AS PER CENT OF QUANTITY ADDED AS CORTISOL-4-C^{14} FROM THE DOG HEART-LUNG AND FROM BLOOD CONTROLS

	Heart	Lung	Blood	Total
Dog 1	12.7	20.5	36.6	69.8
Dog 2	10.0	25.1	30.8	65.9
Incubated control				81
Non-incubated control				77

The recovery values for control specimens are of the order of magnitude usually observed when cortical steroids are extracted from blood or tissue. The approximately 11% average lower recovery from the heart-lung preparations is explained in part by incomplete collection of blood from the heart-lung and connecting tubes.

The activity extracted from the blood of the heart lungs represented about 10 μg. per 100 ml. as cortisol.

Only cortisol was found in the control blood specimens by the chromatographic procedures described.

Two radioactive substances were found in the heart, in the lungs, and in the blood from the heart-lung preparations. The less polar of these substances (Fraction II) when mixed with authentic cortisol showed a precisely similar distribution on the paper chromatogram of radioactivity and of ultraviolet light absorption. Corresponding distribution of radioactivity and ultraviolet light absorption was found after acetylation of the mixture and after oxidation to a 17-ketosteroid. Phenylhydrazine chromogenicity and radioactivity of the mixture were similarly distributed before and after acetylation. This fraction appears to be unaltered cortisol.

The more polar of the two radioactive substances isolated (Fraction I) was mixed with 11 beta, 17 alpha, 20 beta, 21-tetrahydroxy-delta 4-pregnene-3-one. Paper chromatograms showed a precisely similar distribution of radioactivity and ultraviolet light absorption before and after acetylation and after oxidation to a 17-ketosteroid. This fraction appears to be 11 beta, 17 alpha, 20 beta, 21-tetrahydroxy-delta 4-pregnene-3-one.

Table 2 shows the ratios of radioactivity in the 11β, 17α, 20β, 21-tetrahydroxy-Δ^4-pregnene fraction (I) and the cortisol fraction (II) for each tissue. Also shown are calculations of the percentage of total radioactivity added to the preparation found in each fraction in each tissue.

The origins of chromatograms of both control and experimental specimens contained up to 5% of the radioactivity on the strip. The amount of radioactivity remaining at the origin seemed to be correlated with the amount of non-steroidal material in the extract. Several origins were pooled and chromatographed in a formamide:chloroform system for 24 hours. No radioactivity moved from the origin. Pentols have mobilities of 0.25 to 0.85 cm. per hour in this system (2). Pooled origins were subjected to the acetylating procedure and chromatographed for various periods up to five hours in a propylene glycol:toluene system. No movement of radio-

activity from the origin was observed. These experiments indicate that the origins did not contain steroids of polarity equal to or less than that of the pentols and that no readily acetylable steroids were present. The radioactivity at the origins probably represents a chromatographic artefact rather than the presence of additional metabolic products.

The run-off from the initial chromatograms contained one to two per cent of the radioactivity on the paper. The run-offs were rechromatographed in a propylene glycol:n-hexane system for 24 hours and in a propylene glycol:toluene system for 15 hours. The radioactivity remained at the origin in both cases. These experiments indicate the absence of

TABLE 2. DISTRIBUTION IN THE HEART-LUNG OF RADIOACTIVITY BETWEEN 11β, 17α, 20β, 21-TETRAHYDROXY-Δ^4-PREGNENE-3-ONE (FRACTION I) CORTISOL (FRACTION II) AS PER CENT OF TOTAL RADIO-ACTIVITY ADDED AS CORTISOL-4-C^{14}

	Fraction I	Fraction II	Ratio I/II
Dog 1			
Heart	1.0	11.7	0.09
Lung	3.7	16.8	0.22
Blood	5.3	31.3	0.17
Total	10.0	59.8	
Dog 2			
Heart	1.3	8.7	0.15
Lung	4.3	20.8	0.21
Blood	4.9	25.9	0.19
Total	10.5	55.4	

measureable amounts of steroids with the mobility of 17-ketosteroids from the extracts. The phenomenon may represent a chromatographic artefact of a type which has also been observed by others (17).

DISCUSSION

The experiments described above differ in several important respects from previously reported investigations of extrahepatic cortisol metabolism. First, physiological concentrations of steroid have been employed. Second, the isolated heart-lung is a closed system having the advantage of nearly complete recovery of the added substance. Finally, the analytical method described was designed for positive recognition of small quantities of steroid metabolites. Such an approach yields firmer conclusions than one based on decreased recovery of a nonspecific chromogen (8, 12).

In order to investigate the possibility that small quantities of metabolites other than 11β, 17α, 20β, 21-tetrahydroxy-Δ^4-pregnene-3-one had been produced, the extracts of the total specimen of heart, of lung, and of blood of dog 2 were each chromatographed three to four times on multiple papers in order to obtain a purified preparation which could be chromatographed on a single 3 cm. strip. No additional substances were found. However, other metabolites may have been present in quantities too small to

be detected by our methods or labile steroids may have been destroyed by the isolation procedures.

The demonstration that cortisol undergoes reduction of the 20-ketone in the heart-lung preparation relates to the general question: Are the biological actions of the corticosteroids coupled with chemical transformations of these substances? If cortisol acts as a coenzyme in hydrogen transport, one would expect the reduction to be reversible. The possibility should be put to experimental test by presenting C^{14}-labeled 11β, 17α, 20β, 21-tetrahydroxy-Δ^4-pregnene-3-one to a heart-lung preparation. Abelson (18) claims that this steroid has a slight glycogenic action in the adrenalectomized mouse, whereas Glenn *et al.* (3) have been unable to demonstrate such an action. Weak biological activity does not rule out conversion of 11β, 17α, 20β, 21-tetrahydroxy-Δ^4-pregnene-3-one to cortisol, since Glenn *et al.* (3) have demonstrated that potency is determined not only by groups which endow biological activity but also by groups which alter rate of degradation. Reduction of the 20-ketone may prevent the steroid reaching the target site in effective concentration because of rapid rate of degradation. The problem is a complex one and we are not in a position to state whether reduction of cortisol at C-20 by the heart-lung preparation is merely a degradative process or is indeed related to biological action.

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AGE AND CASTRATION IN RELATION TO FATTY LIVER IN THE MALE RAT[1]

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ABSTRACT

At autopsy, 20 of 21 male rats aged 571–821 days had grossly fatty, enlarged livers. By contrast, 24 aged neonatal castrates did not demonstrate this finding. Further, the livers of 24 younger animals, 77–173 days, from comparable treatment groups, were not grossly fatty. Sections were made from all livers. Extracted liver fat and the histologic findings paralleled the gross findings of fatty and non-fatty livers.

The various short-term treatments which some of the rats received prior to sacrifice appeared to have no effect upon hepatic fat. Thus, in these animals, the two factors apparently contributing to the presence of fatty liver were old age and the retention of the gonads until old age.

There is ample evidence in the literature that diet affects liver fat; however, if diet is implicated in these findings, then the lack of fatty liver: a) in the 24 aged neonatal castrates and, b) in one aged testosterone-treated castrate, both warrant explanation.

INTRODUCTION

DURING studies of the effects of age and castration upon reactivity to androgen in male rats it was observed at autopsy that the livers of some aged animals were grossly fatty, while those of others were not. For the original purposes of the study, several groups of animals received various short-term treatments prior to sacrifice. This information is presented so that the reader may know what was done to each animal. However, hepatic fat, with which this report is concerned, appears not to have been affected by these brief treatments.

MATERIALS AND METHODS

Seventy-nine male rats of a locally-inbred derivative of the Osborne-Mendel strain were utilized, 34 being *young* (77-173 days) and 45 *aged* (571–821 days). Thirty-six were castrated on the day of birth using Pfeiffer's method (1); 12 of these that were studied from 91 to 173 days are referred to as *young neonatal castrates*, 24 that were

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studied from 571 to 821 days are referred to as *aged neonatal castrates*. Thirty animals were castrated by abdominal route 3 weeks prior to sacrifice, 16 of these, being 77–147 days old when studied, and referred to as *young castrates*, 14 being 574–814 days old when studied are referred to as *aged castrates*. Thirteen animals remained intact throughout the experiment; 6 studied at 79–138 days are referred to as *young intact animals*, 7 studied at 571–821 days are referred to as *aged intact animals*. Litter mates were used in the several categories whenever possible. All animals were identically caged and fed on Purina Fox Chow and water *ad libitum*. All animals were killed by intrathoracic injection of an excess of Nembutal. At autopsy, the liver was weighed and an approximately 50 mg. sample taken from the tip of the left lobe for routine H and E sections. The livers from 54 animals, comprising 12 young and 8 aged neonatal castrates, 16 young and 9 aged castrates, and 6 young and 3 aged intact animals, were dried for 3 days at 105° F, re-weighed and fat extracted with petroleum benzin.

In addition to castration 3 weeks prior to sacrifice, the short-term treatments included: in 11 aged neonatal castrates, 2 were given 0.15 cc. normal saline intravenously (I.V.) one hour prior to sacrifice, 5 were given 300 μg. aqueous testosterone[4] in 0.15 cc. normal saline I.V. one hour before sacrifice, 4 were given no treatment. All other animals were injected subcutaneously with 0.5 cc. sesame oil, with or without 500 μg. testosterone propionate (T. P.)[5] for 7 consecutive days prior to sacrifice.

RESULTS

At autopsy, 20 of the 21 aged intact animals and aged castrates had large, grossly fatty livers. In contrast, the livers of all of the 24 aged neonatal castrates were of normal size and appearance. None of the livers of the 34 young animals (intact, castrates and neonatal castrates) was either enlarged or fatty. Table 1 gives the findings on fatty liver with ages and information on treatment. The results of liver fat extraction are presented in Table 2. The one aged T.P.-treated castrate which did not have a fatty liver should be noted, as fatty liver was seen in all but one of the aged animals that were not castrated neonatally, and in none of the 24 aged neonatal castrates.

The histologic sections of all livers paralleled the gross observations and extracted fat values. Sections from aged intact animals and aged castrates with and without T.P. showed massive fatty metamorphosis, loss of normal architecture with random hyperplastic liver cords, minimal periportal fibrosis, cytomegaly with vacuolization, "foamy" cytoplasm, amorphous eosinophilic cytoplasmic inclusions, nucleomegaly or pyknosis, an increased number of binucleated cells and chronic passive congestion. The one T.P.-treated aged castrate which had no gross fat showed only moderate congestion and mild hydropic change. Sections from aged neonatal castrates showed no fatty metamorphosis, cytomegaly or nuclear pyknosis; all had

[4] The water-soluble testosterone 17-β, diethylaminoethyl-carbonate hydrochloride was synthesized by the Ciba Pharmaceutical Products, Inc. and supplied through the generosity of Dr. C. H. Sullivan. An additional amount of the same material was generously supplied by Dr. Albert Segaloff of the Alton Oschsner Medical Foundation.

[5] Testosterone propionate was supplied by the courtesy of Ciba Pharmaceutical Products, Inc.

TABLE 1. FATTY LIVER IN AGED AND YOUNG ANIMALS

Treatment (in 0.5 cc. sesame oil for 7 days except when marked*)	Age in days (mean)	Number of animals	Number of animals with fatty liver
Intact male +0.5 cc. S.O.	571–821 (665)	7	7
Adult castrate +0.5 cc. S.O.	574–814 (694)	7	7
Adult castrat +500 μg. T.P.	600–724 (671)	7	6
Neonatal castrate +0.5 cc. S.O.	571–821 (700)	6	0
Neonatal castrate +500 μg. T.P.	600–766 (680)	7	0
Neonatal castrate*	574–765 (666)	11	0
Intact male +0.5 cc. S.O.	79–138 (104)	6	0
Adult castrate +0.5 cc. S.O.	77–147 (118)	8	0
Adult castrate +500 μg. T.P.	96–147 (108)	8	0
Neonatal castrate +0.5 cc. S.O.	91–138 (115)	6	0
Neonatal castrate +500 μg. T.P.	107–173 (140)	6	0

* 5 animals 300 μg. Aqueous Testosterone ×1.
2 animals 0.15 cc. normal saline ×1.
4 animals no treatment.
T.P. = Testosterone Propionate.
S.O. = Sesame Oil.

normal architecture with only occasional patchy areas of mild hydropic change. Liver sections from young animals in all groups were normal in appearance. Representative sections of liver are shown in Figures 1–4.

DISCUSSION

An extensive bibliography relating fatty liver to many pathologic conditions, including dietary abnormalities, is included in *Annales de la Nutrition et de L'Alimentation*, Vol. VII (1953), but no reference was found to the development of fatty livers in aged rats which had received a presumably adequate diet and were not specifically subjected to known hepatotoxins. Lowry, *et al.* stated that there were no pronounced changes in the chemical composition of the liver, including fat, in aged Yale-Cornell and Wistar strain animals (2). Klein and Johnson (3), however, suggested that diets which supported good growth and adequate reproductive function might not meet nutritional requirements in senescent animals and might

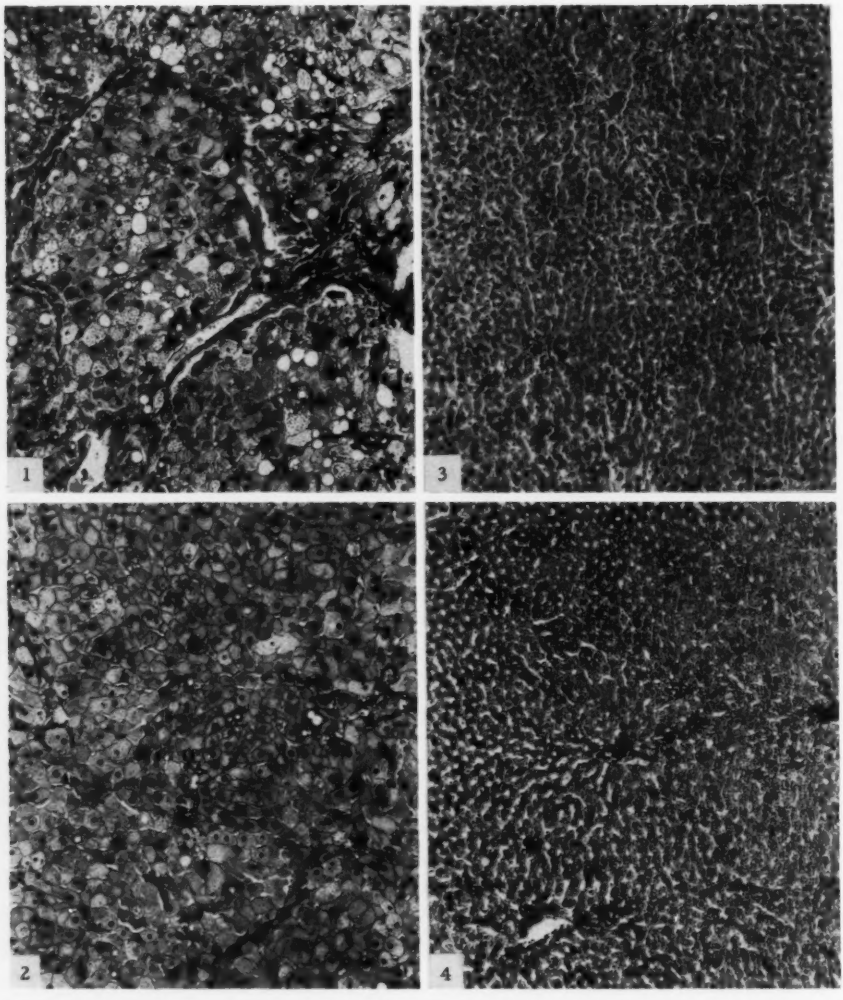
TABLE 2. EXTRACTED LIVER FAT FROM AGED AND YOUNG ANIMALS

Treatment in 0.5 cc. sesame oil for 7 days, except when marked*)	Number of animals	Age in days (mean)	Wt., gms. (mean)	Liver wt. in grams (mean)	Mg. fat/gm. liver (mean)
Intact male +0.5 cc. S.O.	3	571–574 (572)	320–410 (354)	12.464–15.404 (14.113)	140.8–191.8 (166.1)
Adult castrate +0.5 cc. S.O.	4	574–710 (650)	280–370 (313)	12.257–17.309 (14.220)	180.7–204.8 (192.9)
Adult castrate +500 g. T.P.	5	600–724 (658)	328–382 (349)	8.338–16.816 (12.276)	25.2†–219.6 (163.1)
Neonatal castrate +0.5 cc. S.O.	1	571	256	5.153	25.2
Neonatal castrate +500 μg. T.P.	2	600 (600)	280–288 (284)	3.637–5.412 (4.525)	25.8–26.9 (26.4)
Neonatal castrate*	5	658–724 (705)	255–375 (314)	5.481–8.111 (6.414)	17.8–36.8 (27.7)
Intact male +0.5 cc. S.O.	6	79–138 (104)	250–323 (277)	6.918–9.556 (8.101)	13.3–16.8 (14.4)
Adult castrate +0.5 cc. S.O.	8	77–147 (118)	185–312 (248)	4.957–9.938 (7.205)	10.4–17.2 (13.8)
Adult castrate +500 μg. T.P.	8	96–147 (108)	240–300 (278)	7.988–9.793 (8.923)	9.3–15.7 (11.7)
Neonatal castrate +0.5 cc. S.O.	6	91–138 (115)	190–260 (230)	4.780–7.261 (5.861)	13.1–23.7 (17.0)
Neonatal castrate +500 μg. T.P.	6	107–173 (140)	180–250 (218)	5.142–7.820 (6.737)	8.6–12.6 (12.0)

* 3 animals no treatment—2 animals normal saline ×1.
† 1 animal 25.2 mg., see text for discussion.
T.P. = Testosterone Propionate.
S.O. = Sesame Oil.

result in the development of the signs of fatty acid deficiency. Shipley, Chudzik and Györgi (4) stated that castration had no effect upon the development of fatty liver; Hall and Korenchevsky (5) felt that testosterone had a stimulating effect upon the liver, while pre-pubescent castration caused a decrease in liver lobule size. Györgi, Rose and Shipley (6) found that exogenous testosterone had only minimal lipotropic effect; Farber, Koch-Weser and Popper (7) and Farber and Segaloff (8) stated that androgens protect against the development of fatty liver caused by ethionine and that castrated males were highly susceptible to methionine-deficiency fatty liver. The latter investigators found that three androgens of various potencies depressed liver lipid level and hypothesized that this was due to the protein-sparing effect of androgens and perhaps to a relation between testosterone and methionine. Emerson, Zamechik and Nathanson (9) showed that exogenous T.P. aggravated the hepatic and renal lesions produced by high-fat, low-choline diet. In the animals in the present study, fed a presumably adequate diet, neonatal castrates had lower liver fat levels at all ages, the difference being striking in aged animals.

Although we have no explanation for this occurrence, or why it has not been reported by others, several possibilities should be mentioned: The feeding habits of the groups may have differed; there is some suggestion of this as aged intact animals weighed more (mean = 382 gm.) than aged neonatal castrates (mean = 319 gm.), although this was not statistically



Liver sections from male rats following various treatments. Figures at ×130 magnification.

FIG. 1. Liver from aged intact male.

FIG. 2. Liver from aged castrate.

FIG. 3. Liver from aged neonatal castrate.

FIG. 4. Liver from young intact male.

significant. It is possible that these findings in the Osborne-Mendel rat may represent a special case or condition. Mickelsen, Takahashi and Craig (10) reported on the utilization of *ad libitum* feeding of high-fat diets in the production of obesity in 3 strains of rats. They reported that weanling NIH Black rats fed their diet #10,004 attained an average weight of

356 gm. after 25 weeks of the diet; Sprague-Dawley rats weighed 464 gm. and Osborne-Mendel rats 666 gm. after the same time interval. These data may suggest a difference in the handling of ingested fat by the Osborne-Mendel rat.

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THE EFFECT OF NATURAL ESTROGENS AND A SYNTHETIC STEROID ON THE PHOSPHOLIPID METABOLISM OF THE CHICK

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ABSTRACT

Cockerels were treated with equivalent doses of one of three steroids; estrone, estradiol benzoate, or a new synthetic material, methoxy-methyl-estratriene-diol (SC-6924). The steroids increased in identical fashion the plasma phospholipid concentration and turnover rate and the hepatic phospholipid specific activity. In addition, evidence for steroid potentiation of a selective secretion into plasma of an hepatic lecithin fraction of high specific activity is presented. It was concluded that the steroids enhanced the activity of both the synthetic and the catabolic systems associated with hepatic phospholipid metabolism. Furthermore, it was apparent that lecithin synthesis in the liver proceeds more rapidly than does that of the cephalins as a result of the steroid treatment.

IT IS an established fact that estrogens profoundly modify plasma lipid components in the cockerel (1, 2). In this report we have compared two natural estrogens, estrone and estradiol benzoate, and a synthetic steroid, methoxy-methyl-estratriene-diol (SC-6924),[1] in their effects upon the rates of incorporation of inorganic P^{32} into the phospholipids of liver and plasma.

METHODS

Four-week old cockerels fed a starter mash were divided into four groups of four birds each and were treated intramuscularly as follows: (I) control birds given oil vehicle alone; (II) estrone 15 mg./kg.; (III) estradiol benzoate 3 mg./kg.; (IV) SC-6924 60 mg./kg. These doses of the steroids in oil were shown in preliminary experiments to bring about equivalent increases of plasma phospholipid levels at the time interval studied. Twenty-six hours after the administration of test compounds, when plasma phospholipid levels remained relatively constant and a new steady state had been established, the fasted birds were given intravenously about 1 mc. P^{32} as sodium phosphate. Plasma samples were drawn and liver biopsies were performed at 4, 8, and 20 hours after the administration of P^{32}. Plasma and homongenized liver samples were extracted by the chloroform-methanol methods of Folch, *et al.* (3, 4). Phospholipid phosphorus in the extracts was estimated by the method of King (5). Fractionation of plasma extracts into lecithins (choline-containing phospholipids) and cephalins (non-

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[1] SC-6924 is 3-Methyoxy-16α-methyl-1, 3, 5 (10) estratriene 16β, 17β-diol a synthetic steroid synthesized by Dr. David A. Tyner of the Chemical Research Division of G. D. Searle & Co. This compound (Manvene) has the property of profoundly modifying the lipid metabolism of the chick but exhibits only weak estrogenic properties.

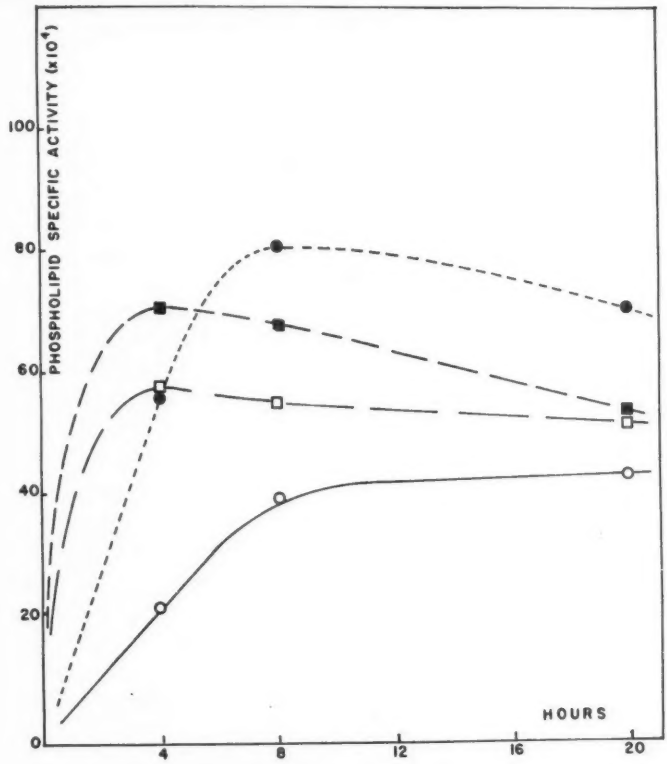

FIG. 1. Liver and plasma phospholipid specific activity-time curves. Symbols: Open circle plasma of control chicks; open box, liver of control chicks; closed circle, plasma of estradiol benzoate treated birds; closed box liver of estradiol benzoate treated birds. The phospholipid specific activity is calculated as the per cent of administered P^{32} recovered per mg. of phospholipid. The abscissa indicates the time elapsed after the administration of the radioisotope.

choline containing phospholipids) was carried out on pooled samples from Groups I and III by the adsorption of the lipid extracts on MgO and the subsequent elution of lecithins by methanol (6). The radioactivity of the extracts was measured on direct mounts with an end-window geiger counter.

RESULTS

Figure 1 gives the specific activity-time curves of the control birds and curves representative of the test groups. Since the curves for all steroid treated groups were nearly identical, only those for estradiol benzoate have been illustrated.[2] The liver phospholipid specific activity in the con-

[2] The maximum values for liver and plasma specific activities of all groups at 4 and 8 hours respectively have been listed in Table 1.

TABLE 1. THE EFFECTS OF STEROID TREATMENT ON PLASMA PHOSPHOLIPID METABOLISM IN THE CHICK

Group	Treatment	Mean body weight (gm.)	Mean plasma phospholipid			Mean maximum specific activity*	
			Concentration at 4 hrs. (mg./ml.)	Turnover time (hrs.)	Turnover rate (mg./hr./ml.)	Liver (4 hrs.)	Plasma (8 hrs.)
I	Oil Control	433	1.94	6.67	0.291	5.74×10^{-3}	4.40×10^{-3}†
II	Estrone, 15 mg./kg.	371	5.55	2.69	2.03	8.38×10^{-3}	9.09×10^{-3}
III	Estradiol Benzoate, 3 mg./kg.	391	4.49	1.85	2.43	7.10×10^{-3}	8.06×10^{-3}
IV	SC-6924, 60 mg./kg.	385	4.77	2.18	2.19	7.65×10^{-3}	7.76×10^{-3}

* Specific activity is calculated as the per cent administered P^{32} recovered per mg. phospholipid.
† The value listed is the maximum control specific activity observed at 20 hours.

trol group rose rapidly to a maximum at 4 hours and then gradually receded while that of plasma continued to rise toward an intersection with the hepatic curve at some time later than 20 hours.

The specific activity-time curves for both the liver and the plasma of the treated birds significantly exceeded the comparative control data. In addition, the rates of disappearance of the labeled phospholipid (as indicated by the slopes of these curves from 8–20 hours) was greater in the treated birds than in the controls. An interesting and unexpected observation was that in the treated group the maximum plasma value exceeded that of the liver, though this relationship was not seen in the control curves.

We have calculated the plasma phospholipid turnover rates for the four groups according to the method of Zilversmit *et al.* (7) using the rapidly rising linear phase of the curves and assuming hepatic phospholipid to be the immediate precursor of the plasma component. These results are given in Table 1 and indicate a nearly ten-fold increase in plasma phospholipid turnover rates in the steroid treatment groups when they are compared with the control.

Fractionation of the plasma phospholipids on MgO (Table 2) showed that the specific activity of the lecithins increased more rapidly than did that of the cephalins, thus contributing a greater proportion of the plasma radioactivity in the first 8 hours. At 20 hours the specific activity of the

TABLE 2. FRACTIONATION OF POOLED PLASMA EXTRACTS INTO LECITHINS AND CEPHALINS

Treatment	Time (hrs.)	Plasma phospholipid specific activity*	
		Lecithins	Cephalins
Control	4	4.64×10^{-3}	4.62×10^{-3}
	8	14.0×10^{-3}	6.20×10^{-3}
	20	6.0×10^{-3}	7.40×10^{-3}
Estradiol benzoate	4	21.0×10^{-3}	6.95×10^{-3}
	8	33.5×10^{-3}	10.5×10^{-3}
	20	11.2×10^{-3}	21.5×10^{-3}

* Specific activity defined in Table 1.

cephalins had exceeded that of the lecithins. The early differences between the specific activities of the two fractions were more pronounced in the steroid treated birds than in the controls.

DISCUSSION

The data presented here indicate that the three steroids examined had identical effects in terms of increasing the plasma phospholipid turnover rate, the plasma phospholipid concentration, and the intensity of hepatic phospholipid formation. In addition, an equivalent increase in the rate of disappearance of both hepatic and plasma phospholipids was observed in the treated animals. Apparently these compounds modify hepatic phospholipid metabolism in identical fashions.

These results confirm the hypothesis that we advanced previously (8), namely, that steroids of this type enhance the activity of both the synthetic and the catabolic systems associated with hepatic phospholipid metabolism. In the present data the steroid-induced increase in turnover rate while a steady state existed was indicative of a stimulation of the incorporation of P^{32} into the plasma lipid as well as an enhancement of the disappearance of the labeled material from the circulation.

Of particular interest is the observation that under the influence of the steroid plasma phospholipid specific activity rose to a value greater than that of the liver. This might indicate either an extrahepatic source of plasma phospholipid or a phospholipid moiety in liver of high specific activity that was selectively secreted to the plasma. The possibility of an extrahepatic source of a highly reactive phospholipid component seems slight since we have shown recently (8) the ineffectiveness of SC-6924 to modify the plasma phospholipid specific activity in the hepatectomized chick. Fractionation of the plasma phospholipids indicated that in the treated birds the initial high specific activity found in this lipid fraction was due principally to the high activity of the choline-containing phospholipids. It should be recalled that the ratio of lecithins to cephalins found in the liver is 1.03 while in the plasma it is 3.76 (9). From these facts we suggest that in the birds treated with these steroids the plasma phospholipid specific activities rose to values higher than those of the liver because there was an initial selective release to the plasma of a high specific activity fraction of hepatic lecithins. Further work is needed to clarify this problem and substantiate this hypothesis.

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BIOASSAY OF INTERSTITIAL CELL-STIMULATING HORMONE IN THE HYPOPHYSECTOMIZED MALE RAT BY THE VENTRAL PROSTATE TEST[1]

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ABSTRACT

Several factors that could affect the assay for ICSH in the hypophysectomized male rat by the prostate test have been investigated. With respect to route of injection, it was found that the hormone solution should be administered subcutaneously rather than intraperitoneally for more quantitative determinations. Moreover, for the assay of purified preparations of ICSH, rats of the Long-Evans and the Sprague-Dawley strains were found to be equally satisfactory with respect to consistency and sensitivity. On the other hand, it was shown that whereas the administration of either growth hormone or lactogenic hormone concurrently with ICSH had no influence on the prostatic response of the Long-Evans rat to the latter hormone, in the Sprague-Dawley rat both of the former hormones were complicating factors in the assay. The evidence in support of the interpretation that lactogenic hormone intervenes at the level of the testis and that growth hormone intervenes at the level of the ventral prostate as well as of the testis is discussed.

STIMULATION of the sex accessories of the hypophysectomized rat, including the ventral prostate, can be effected by non-gonadotropic hormones of the anterior pituitary gland (1), and it is to be anticipated that these hormones could influence the assay for interstitial cell-stimulating hormone (ICSH) in which the weight of the ventral prostate is used as a measure of the gonadotropic activity. It is also true that hypophysectomy results in greater prostatic atrophy than does castration (2) and that treatment with growth hormone restores the prostate of the hypophysectomized-castrated male to the castrate condition (1, 3). Furthermore, the administration of growth hormone to the hypophysectomized-castrated Sprague-Dawley rat restores the capacity of this animal to respond to exogenous androgen (3). On the other hand, the hypophysectomized-castrated Long-Evans rat responds fully to exogenous testosterone and no enhancement in the response is observed following the administration of growth hormone (4). It has also been reported that lactogenic hormone likewise influences the response of the ventral prostate both to androgen

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(5, 6) and to ICSH (7). In our earlier studies it was demonstrated that lactogenic hormone is ineffective as a synergist when given in combination with testosterone propionate (4). This communication is concerned with factors that could affect the assay of ICSH by means of the prostate test, including the effect of growth hormone, lactogenic hormone and their combination, on the assay, the influence of the route of injection on the response, and the sensitivity of the Long-Evans strain as compared to the Sprague-Dawley.

METHODS

Hypophysectomized male rats of the Long-Evans and Sprague-Dawley strains were injected with ICSH following two different post-operative intervals. In the first series, the animals were hypophysectomized at 21 days of age, and only 2 days intervened between the time of operation and the first injection of ICSH. In the second series, the animals were hypophysectomized at 28 days of age and the first injection of ICSH was given 14 days after the operation. In every instance, the ICSH solution was injected once daily for a period of 4 days and the rats were sacrificed 24 hrs. after the last injection. The growth and lactogenic hormones were given subcutaneously, once daily, either during the entire postoperative period or throughout only a part of it. At autopsy, the ventral prostate, seminal vesicles and right testis were removed and weighed.

The ICSH was isolated from sheep pituitary glands by the new procedure recently described (8); the "C" material is a partially-purified preparation used as a standard in our laboratory and the "F" material is a highly purified preparation that is free from any detectable contamination and displays a high degree of molecular homogeneity. The lactogenic hormone was also prepared from sheep pituitaries (9) and the growth hormone was prepared from bovine pituitaries (10).

RESULTS

Effect of Injection Route. ICSH was injected either intraperitoneally or subcutaneously into 21-day old hypophysectomized male rats; the results are summarized in Table 1. From the data it is apparent that ICSH, when administered intraperitoneally, is effective at lower dose levels than when it is administered subcutaneously. For example, a total dose of 0.010 mg. produced a ventral prostate weight of 14.2 mg. when injected by the former route, whereas by the latter route 0.015 mg. was required to effect a similar increase. On the other hand, no difference in response was evident with dose levels of 0.025, 0.030, and 0.060 mg. Mathematical analysis of the results obtained with the four dose levels tested revealed that the slope for the regression line was 7.9 for the intraperitoneal and 12.4 for the subcutaneous route, and that the calculated indices of precision (λ) were 0.51 and 0.19, respectively. Increases in the weights of the testes closely paralleled the increases observed in the ventral prostate at the dose levels that ordinarily would be selected for assay; on the other hand, the seminal vesicles were quite unresponsive and no significant increase in their weight was observed at a dose level 300 times the level that effected a 50% increase in the weight of the ventral prostate.

TABLE 1. THE INFLUENCE OF THE ROUTE OF INJECTION ON THE PROSTATIC RESPONSE OF THE HYPOPHYSECTOMIZED* LONG-EVANS RAT TO ICSH

Route of injection	Total dose	No. of rats	Body weight		Organ weights	
			Onset	Autopsy	Ventral prostate	Right testis
	mg.		gm.	gm.	mg.	mg.
	0	8	45	47	7.9±0.3†	66±3
Intraperitoneal	0.010	7	49	52	14.2±1.5	76±5
	0.015	8	47	51	19.7±2.4	82±3
	0.025	6	47	51	16.8±0.9	81±5
	0.030	6	45	49	20.3±2.3	84±5
	0.060	6	51	54	20.6±0.7	97±5
Subcutaneous	0.010	7	52	54	11.0±0.5	78±3
	0.015	7	47	51	15.3±1.2	85±6
	0.025	6	52	55	16.8±0.4	94±8
	0.030	8	44	48	18.7±0.8	83±3
	0.060	6	50	52	21.3±1.2	90±6

* All animals were hypophysectomized at 21 days of age and autopsied at 27 days. Single daily injections of fraction "C" were given for a period of 4 days, beginning at 23 days of age.
† Mean ± standard error.

Effect of Animal Strain. A comparison was also made between prostatic responses in the Long-Evans and the Sprague-Dawley strains. As can be seen from Table 2, similar increases in the weight of the ventral prostate above that of the controls were obtained with total dose levels of 0.001, 0.010 and 0.060 mg. of ICSH injected intraperitoneally. When the slopes of the dose response curves in the two strains were calculated, a value of 9.9 was obtained for the Long-Evans strain and a value of 8.2 for the Sprague-Dawley strain, with a slightly smaller deviation about the regression line in the latter case ($\lambda = 0.56$ and 0.47, respectively).

Effect of Growth Hormones and Lactogenic Hormone. From the data summarized in Tables 3, 4 and 5, it is evident that the administration of a daily dose of 0.035 mg. of lactogenic hormone did not influence either the

TABLE 2. ASSAY OF ICSH BY THE PROSTATE TEST IN HYPOPHYSECTOMIZED* MALE RATS OF THE LONG-EVANS AND SPRAGUE-DAWLEY STRAINS

Strain	Total dose	No. of rats	Body weight		Organ weights		
			Onset	Autopsy	Ventral prostate	Seminal vesicles	Right testis
	mg.		gm.	gm.	mg.	mg.	mg.
Long-Evans	0	5	50	55	8.5±0.6†	7.4±0.8	66±3
	0.001	5	48	55	13.6±1.0	6.9±0.4	76±6
	0.010	8	49	56	20.1±2.0	7.2±0.4	84±8
	0.060	8	52	59	30.9±2.3	9.2±0.6	109±7
	0.300	7	51	57	29.0±3.1	8.4±0.8	114±8
Sprague-Dawley	0	6	44	42	6.7±0.7	7.1±0.3	71±4
	0.001	8	44	41	9.9±1.0	7.9±0.5	75±5
	0.010	8	45	42	18.4±1.2	9.6±0.6	98±5
	0.060	8	43	42	24.5±1.7	8.1±0.6	92±4

* All animals were hypophysectomized at 21 days of age and autopsied at 27 days. Single daily injections of fraction "C" were given intraperitoneally for a period of 4 days, beginning at 23 days of age.
† Mean ± standard error.

TABLE 3. EFFECT OF GROWTH HORMONE AND LACTOGENIC HORMONE ON THE ASSAY OF ICSH BY THE PROSTATE TEST IN MALE RATS HYPOPHYSECTOMIZED AT 21 DAYS*

Strain	Treatment	Daily dose	No. of rats	Body weights		Organ weights	
				Onset	Autopsy	Ventral prostate	Right testis
		mg.		gm.	gm.	mg.	mg.
Long-Evans	Saline	0	8	45	47	7.9 ± 0.3†	66 ± 3
	ICSH, "F" Fraction	0.0005	8	51	54	12.8 ± 0.8	84 ± 7
	ICSH, "C" Fraction	0.005	6	48	52	18.7 ± 1.3	93 ± 7
	Growth Hormone	0.035	8	55	59	10.9 ± 0.6	82 ± 5
	Lactogenic Hormone	0.035	8	51	56	7.9 ± 0.4	64 ± 3
	ICSH, "F" Fraction + Growth Hormone	0.0005 + 0.035	8	51	55	13.0 ± 1.2	82 ± 5
	ICSH, "C" Fraction + Growth Hormone	0.005 + 0.035	6	49	57	21.3 ± 2.2	95 ± 4
	ICSH, "F" Fraction + Lactogenic Hormone	0.0005 + 0.035	8	50	53	13.0 ± 1.4	90 ± 10
	ICSH, "C" Fraction + Lactogenic Hormone	0.005 + 0.035	6	49	54	19.9 ± 2.8	106 ± 8
	ICSH, "F" Fraction + Growth Hormone + Lactogenic Hormone	0.0005 + 0.035 + 0.035	8	54	59	15.8 ± 1.0	88 ± 5
	ICSH, "C" Fraction + Growth Hormone + Lactogenic Hormone	0.005 + 0.002 + 0.002	7	50	54	19.7 ± 2.4	103 ± 11
	ICSH, "C" Fraction + Growth Hormone + Lactogenic Hormone	0.005 + 0.035 + 0.035	6	49	57	19.8 ± 2.6	101 ± 11
Sprague-Dawley	Saline	0	6	44	42	6.7 ± 0.7	71 ± 4
	ICSH, "C" Fraction	0.005	7	49	54	15.1 ± 0.9	108 ± 10
	Growth Hormone	0.002	8	50	58	8.7 ± 0.6	93 ± 11
	Lactogenic Hormone	0.002	8	50	55	8.3 ± 0.6	90 ± 10
	ICSH, "C" Fraction + Growth Hormone	0.005 + 0.002	7	49	58	22.3 ± 1.9	138 ± 12

* Single daily injections were given for a period of 4 days beginning at 23 days of age with autopsy at 27 days. All hormones were administered subcutaneously.

† Mean ± standard error.

TABLE 3.—*Continued*

Strain	Treatment	Daily dose	No. of rats	Body weights		Organ weights	
				Onset	Autopsy	Ventral prostate	Right testis
	ICSH, "C" Fraction + Growth Hormone	0.005 + 0.035	7	55	64	26.3 ± 0.9	136 ± 8
	ICSH, "C" Fraction + Lactogenic Hormone	0.005 + 0.002	8	50	55	19.7 ± 1.0	126 ± 11
	ICSH, "C" Fraction + Lactogenic Hormone	0.005 + 0.035	8	57	62	24.2 ± 0.7	150 ± 14
	ICSH, "C" Fraction + Growth Hormone + Lactogenic Hormone	0.005 + 0.002 + 0.002	7	59	65	24.1 ± 2.1	163 ± 18

prostatic or the testicular response of the hypophysectomized Long-Evans rat to exogenous ICSH. Neither did growth hormone when administered together with ICSH to animals that were hypophysectomized at 21 days of age and submitted to hormonal treatment 2 days later. On the other hand, growth hormone when administered in a daily dose of 0.100 mg. from the day following hypophysectomy to the time of autopsy 18 days later, did affect the weights of both organs; comparable results were obtained when the injections were begun 7 days after hypophysectomy and continued for a period of 11 days (Table 5). At best, the increases in the weights of the organs represent an additive effect on the part of the growth hormone and the ICSH, and there is no indication of any synergism between the two.

In contrast to the Long-Evans strain, animals of the Sprague-Dawley strain showed an enhancement in their prostatic response to ICSH when either growth hormone or lactogenic hormone was administered along with the ICSH. This was especially evident in the rats hypophysectomized at 21 days of age and injected with ICSH 2 days postoperatively. As can be seen from Table 3, the weight of the ventral prostate was significantly larger in the animals injected with 0.002 mg. of either lactogenic hormone or growth hormone plus 0.005 mg. of ICSH than in the animals injected with 0.005 mg. of ICSH alone; a similar enhancement was evident in the testicular response. However, different results were obtained with lactogenic hormone and growth hormone when the rats were hypophysectomized at 28 days of age and when the injections of ICSH were not begun until 14 days after the operation. Under these conditions, the animals hypophysectomized at 28 days of age and treated with a daily dose

TABLE 4. EFFECT OF MAINTENANCE TREATMENT WITH GROWTH HORMONE OR LACTOGENIC HORMONE ON THE PROSTATIC RESPONSE OF THE HYPOPHYSECTOMIZED MALE RAT* TO ICSH

Strain	Treatment	Daily dose	No. of rats	Body weights		Organ weights		
				Onset	Autopsy	Ventral prostate	Seminal vesicles	Right testis
				gm.	gm.	mg.	mg.	mg.
Long-	Saline	0	6	86	91	8.4 ± 0.5†	9.4 ± 0.6	85 ± 4
Evans	ICSH, "F" Fraction	0.025	6	76	88	8.0 ± 0.6	7.9 ± 0.3	93 ± 6
	ICSH, "F" Fraction	0.075	6	88	91	11.7 ± 0.7	11.8 ± 1.0	124 ± 4
	ICSH, "F" Fraction	0.250	6	74	85	9.5 ± 0.4	10.6 ± 0.6	107 ± 8
	Growth Hormone	0.100	6	81	112	11.5 ± 0.5	10.2 ± 0.5	101 ± 8
	Lactogenic Hormone	0.100	5	81	90	7.2 ± 0.6	8.04 ± 0.3	79 ± 6
	ICSH + Growth Hormone	0.025 + 0.100	6	81	119	13.6 ± 1.1	12.6 ± 1.3	118 ± 9
	ICSH + Growth Hormone	0.075 + 0.100	6	76	118	13.9 ± 0.7	14.4 ± 1.1	123 ± 8
	ICSH + Growth Hormone	0.250 + 0.100	5	77	115	13.6 ± 1.3	15.3 ± 2.2	143 ± 10
	ICSH + Lactogenic Hormone	0.025 + 100	5	80	92	10.5 ± 0.7	9.0 ± 1.1	100 ± 8
	ICSH + Lactogenic Hormone	0.250 + 0.100	6	76	85	10.2 ± 1.0	8.8 ± 0.6	111 ± 8
Sprague-	Saline	0	8	89	83	7.8 ± 0.5	10.2 ± 0.7	114 ± 9.0
Dawley	ICSH, "F" Fraction	0.075	9	88	83	9.8 ± 0.7	13.5 ± 2.1	147 ± 16.8
	Growth Hormone	0.010	5	86	87	8.2 ± 0.2	10.6 ± 0.7	110 ± 5.2
	Growth Hormone	0.100	8	91	114	18.0 ± 1.0	13.7 ± 0.6	171 ± 13.3
	Lactogenic Hormone	0.100	7	86	83	8.0 ± 0.5	10.4 ± 1.1	105 ± 9.0

* Animals were hypophysectomized at 28 days and sacrificed at 47 days of age. Growth hormone and lactogenic hormone were injected once daily, subcutaneously, throughout the post-operative interval, and ICSH was injected once daily, beginning at 43 days of age.

† Mean ± standard error.

TABLE 4.—*Continued*

Strain	Treatment	Daily dose	No. of rats	Body weights		Organ weights		
				On-set	Au-topsy	Ventral prostate	Seminal vesicles	Right testis
	ICSH, "F" Fraction + Growth Hormone	0.075 + 0.010	6	97	97	13.5±0.5	17.3±2.1	159±13.7
	ICSH, "F" Fraction + Growth Hormone	0.075 + 0.100	10	91	118	39.3±2.7	34.1±3.6	247±13.7
	ICSH, "F" Fraction + Lactogenic Hormone	0.075 + 0.100	9	92	86	12.4±0.5	13.5±0.8	151± 9.7
	ICSH, "F" Fraction + Growth Hormone + Lactogenic Hormone	0.075 + 0.100 + 0.100	10	95	117	51.1±5.6	36.1±3.9	290±19.2
	ICSH, "F" Fraction + Growth Hormone‡	0.075 + 0.100	8	99	96	13.1±0.5	18.3±1.4	169±11.8

‡ This group was treated differently from the other groups in that growth hormone as well as ICSH was withheld until day 43.

of 0.100 mg. of lactogenic hormone from the time of operation to autopsy were only slightly more responsive to ICSH than were the non-maintained controls (Table 4). If the group maintained with lactogenic hormone is compared to a similar group given growth hormone throughout the post-operative period, it is apparent that growth hormone was considerably more effective than lactogenic hormone in enhancing the prostatic response; in fact, growth hormone administered at a daily dose of 0.100 mg. for 18 days promoted a greater increase in the weight of the ventral prostate than did 0.075 mg. of ICSH injected from day 15 through day 18. On the other hand, only in the presence of exogenous or endogenous growth hormone was lactogenic hormone capable of exerting a significant influence on the results.

DISCUSSION

In our experience, the hypophysectomized male rat of the Long-Evans strain has been a satisfactory animal for assaying preparations of ICSH

TABLE 5. EFFECT OF PRIMING TREATMENT WITH GROWTH HORMONE OR LACTOGENIC HORMONE ON THE PROSTATIC RESPONSE OF THE HYPOPHYSECTOMIZED LONG-EVANS RAT* TO ICSH

Treatment	Daily dose	No. of rats	Body weight		Organ weights		
			Onset	Autopsy	Ventral prostate	Seminal vesicles	Right testis
	mg.		gm.	gm.	mg.	mg.	mg.
Saline	0	6	86	91	8.4 ± 0.5†	9.4 ± 0.6	85 ± 4
ICSH	0.025	6	76	88	8.0 ± 0.6	7.9 ± 0.3	93 ± 6
ICSH	0.075	6	88	91	11.7 ± 0.7	11.8 ± 1.0	124 ± 7
Growth Hormone	0.100	6	88	116	11.2 ± 0.9	12.9 ± 1.0	106 ± 8
Lactogenic Hormone	0.035	6	89	94	8.0 ± 0.4	8.5 ± 0.3	91 ± 2
Lactogenic Hormone	0.175	5	75	85	7.1 ± 0.3	7.5 ± 0.5	80 ± 3
ICSH + Growth Hormone	0.025 + 0.100	6	72	90	9.8 ± 0.9	11.7 ± 1.2	89 ± 7
ICSH + Growth Hormone	0.075 + 0.100	6	75	95	10.5 ± 1.3	9.7 ± 1.3	100 ± 9
ICSH + Lactogenic Hormone	0.075 + 0.035	6	72	73	7.4 ± 0.4	8.1 ± 0.9	80 ± 9
ICSH + Lactogenic Hormone	0.075 + 0.175	6	73	83	8.0 ± 0.8	7.2 ± 0.5	99 ± 7
ICSH + Growth Hormone + Lactogenic Hormone	0.025 + 0.100 + 0.035	6	71	90	9.3 ± 0.5	8.8 ± 0.6	92 ± 9
ICSH + Growth Hormone + Lactogenic Hormone	0.075 + 0.100 + 0.035	4	72	91	8.7 ± 0.8	8.0 ± 0.7	96 ± 8

* Animals were hypophysectomized at 28 days and sacrificed at 47 days of age. Growth hormone and lactogenic hormone were injected once daily, subcutaneously, beginning at 35 days of age; ICSH "F" fraction was injected once daily, intraperitoneally, beginning at 43 days of age.

† Mean ± standard error.

when the weight of the ventral prostate is used as a measure of the activity and the response is represented as a logarithmic function of the dose. Routinely, the regimen recommended by Greep, *et al.* (11) has been followed; i.e., the animals are hypophysectomized at 21 days of age and the hormone solution is injected once daily beginning at 23 days, for a period of 4 days. For more quantitative results, the solutions should be injected subcutaneously rather than intraperitoneally. Although the hormone is effective at a lower dose level when injected intraperitoneally rather than subcutaneously, in this test as well as in the histological test that uses repair of the

interstitial tissue of the ovary as a criterion (12), this is not a serious consideration. The sensitivity of the assay is such that a total dose level of from 0.010 to 0.060 mg. is sufficient for assay purposes when the subcutaneous route is employed. As reported previously (12), the seminal vesicles of the Long-Evans rat are quite unresponsive to the hormone and are therefore of little use for assaying ICSH; at a dose level 300 times that required to effect a 50% increase in the weight of the ventral prostate, no increase in the weight of the seminal vesicles was observed. No differences between rats of the Long-Evans and the Sprague-Dawley strains were observed with respect to the responsiveness of either the ventral prostate and testis or the seminal vesicles.

Whereas hypophysectomized rats of either strain would be equally satisfactory for the assay of purified ICSH, those of the Long-Evans strain would seem to be better suited for testing crude preparations that might contain growth hormone or lactogenic hormone. Neither growth hormone, lactogenic hormone, nor combinations of the two alter the prostatic response of the Long-Evans rat to exogenous ICSH. Similarly, no effect was observed previously when these hormones were administered together with testosterone propionate to the hypophysectomized-castrated animal (4), and it may be concluded that growth hormone and lactogenic hormone do not influence the assay for ICSH in the hypophysectomized Long-Evans rat by means of the prostatic method, by intervening at the level of either the testis or the ventral prostate. Finally, it should be added that follicle-stimulating hormone (FSH) likewise does not affect the assay (13).

In the hypophysectomized Sprague-Dawley rat, on the other hand, the prostatic response may not be a valid measure of the ICSH activity if growth hormone or lactogenic hormone is present in the sample to be tested. Since as little as 0.002 mg. of either of the latter hormones injected once daily along with the ICSH effected a significant enhancement of the response of the animal hypophysectomized at 21 days and since this enhancement represents a 200–300% increase in the estimate for the gonadotropin activity, it is evident that assays carried out with crude preparations will provide a questionable measure of the ICSH actually present. Hence, as mentioned above, the Sprague-Dawley rat is not as useful as the Long-Evans rat for the quantitative assay of ICSH activity by the prostate test.

The results obtained in the Sprague-Dawley strain with lactogenic hormone and ICSH confirm those published earlier by Segaloff, *et al.* (7). Furthermore, the fact that we have not been able to detect any synergism between exogenous testosterone propionate and lactogenic hormone with respect to the sex accessories in the hypophysectomized-castrated animal (4, 14) suggests that lactogenic hormone probably influences the assay by its effect on the testis when administered together with ICSH. There is disagreement as to whether or not growth hormone also acts synergistically with ICSH. In order to explain the effects that he obtained in his investiga-

tions, Segaloff has suggested that the enhancement effected by growth hormone preparations can probably be attributed to the lactogenic contaminant present in the preparations (7). The results reported here cannot be interpreted in this way. First of all, growth hormone was in every instance more effective than lactogenic hormone in enhancing the prostatic response to ICSH and, secondly, only in combination with growth hormone did lactogenic hormone exert a significant influence on the results when an interim of 14 days had elapsed prior to the first injection of ICSH. Thus, it has again been demonstrated that the administration of growth hormone permits the hypophysectomized animal to respond more effectively to another exogenous hormone (1, 4). Furthermore, in view of the fact that the prostatic response to exogenous testosterone is enhanced when growth hormone is administered in conjunction with the androgen, we believe that growth hormone itself does affect the assay for ICSH and that it probably intervenes at the level of both the ventral prostate and the testis. The effects of ICSH, growth hormone and lactogenic hormone when administered alone and in combination are consistent with the interpretation that has been advanced.

Acknowledgment

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C^{14} LABELED BACTERIAL POLYSACCHARIDE AND ADRENAL CORTICAL FUNCTION IN THE GUINEA PIG[1,2]

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ABSTRACT

The 17-hydroxycorticosteroids in plasma and bile and the ascorbic acid content of the adrenal have been studied following the administration of bacterial polysaccharide to the guinea pig. A single intravenous injection of the polysaccharide complex from *K. pneumoniae* evokes an elevation in the free 17-OHCS of plasma and bile which lasts 6 to 12 hours. The 17-OHCS response varies directly with the quantity of polysaccharide. The ascorbic acid content of the adrenal is decreased for a longer period of time. After several daily injections of polysaccharide the adrenal ascorbic acid does not return to control range for several days.

The increased adrenal 17-hydroxysteroidogenesis following the injection of a large amount of polysaccharide declines while there is abundant polysaccharide in the plasma and while the adrenal is capable of responding to ACTH.

The presence of bacterial polysaccharide in plasma when the plasma 17-OHCS have returned to normal range is discussed in relation to the pathologic effects of the bacterial polysaccharides.

PREVIOUS studies have shown that the highest tissue concentration of C^{14} after the injection of labeled polysaccharide complexes from *K. pneumoniae* is in the adrenal cortex (1, 2). The C^{14} remains for at least two months in this organ (1). Some of the labeled material in the adrenal retains the haptenic qualities of the original polysaccharide (2), but the exact chemical nature of all the C^{14} moieties in the adrenal is not yet known. Pretreatment by daily injections of cortisone markedly reduces the uptake of C^{14} from the labeled polysaccharide complex (3). This inhibitory effect of cortisone, however, is abolished by concomitant pretreatment with ACTH (3). Such observations suggest that the uptake of C^{14} from labeled polysaccharide may be related to adrenal steroidogenesis.

In the present investigation the effects of *K. pneumoniae* polysaccharide upon adrenocortical function are explored by means of the adrenal ascorbic acid content and the concentration of 17-hydroxycorticosteroids (17-OHCS) in plasma and bile of the guinea pig. Four major aspects are in-

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vestigated: a) the relation of adrenal ascorbic acid content to the concentration of 17-OHCS in plasma, b) the rapidity and duration of plasma 17-OHCS elevation after the injection of different quantities of polysaccharide and of polysaccharide-containing plasma transfused from other animals, c) the clearance from plasma of various quantities of polysaccharide and d) the effect of pretreatment with polysaccharide on the plasma 17-OHCS response to subsequent injection of ACTH or of polysaccharide.

These studies were done in order to evaluate whether the presence of metabolites from bacterial polysaccharides affects the function of the adrenal cortex.

METHODS AND MATERIALS

Guinea pigs weighing 200 to 250 gm. at the initiation of the experiment were fed Purina Rabbit Pellets and a daily supplement of cabbage. The preparation of non-lethal, haptenic polysaccharide-complex used in these experiments has been described in previous publications (4, 5). Unlabeled and C^{14} labeled (2) bacterial polysaccharide were prepared as a 1% solution in 0.85% sodium chloride, autoclaved, and introduced into the transilluminated ear vein. Determination of C^{14} in plasma was carried out as previously described (2).

17-OHCS were determined by the technique of Eik-Nes (6) and ascorbic acid by the method of Roe and Kuether (7). When normal guinea pigs were anesthetized in a saturated ether atmosphere, the plasma 17-OHCS values were found to be higher than when the animals were sacrificed and blood removed by cardiac puncture. Several methods were used to sacrifice the animals: a) cranial blow, b) cranial blow with immediate clamping of the cervical area, and c) cervical clamping. The latter procedure consistently gave the narrowest range of 17-OHCS in normal animals and was used thereafter.

Animals were sacrificed at various intervals after the intravenous injection of the polysaccharide. The blood, drawn from the heart into a syringe containing heparin, was immediately centrifuged and the plasma removed. Autopsies were performed at once. Bile, if present, was aspirated from the gall bladder. A portion of the adrenal was detached, weighed and used for the determination of ascorbic acid content.

Intravenous injections of the polysaccharide (1 mg./100 gm. body weight) were given daily for 1, 2, 3, and 5 days and the 17-OHCS and adrenal ascorbic acid were determined at various intervals after the last injection of polysaccharide.

Guinea pigs were given C^{14} labeled polysaccharide (2 mg./100 gm.) and sacrificed 1, 3, and 6 hours later. The amount of polysaccharide in the plasma samples was estimated by its C^{14} concentration. The pooled plasma from each interval was injected intravenously in 0.5 ml. quantities into normal guinea pigs. Plasma 17-OHCS were determined in these animals at various periods after the administration of the plasma containing the radioactive polysaccharide. Control animals were given normal pooled plasma in a similar fashion.

Thirty animals were given the bacterial polysaccharide intravenously (1 mg./100 gm. body weight) and 6, 9 and 12 hours afterwards these animals received intravenous ACTH (0.5 I.U./100 gm.).

RESULTS

Plasma 17-Hydroxycorticosteroids in Control Animals

The clamping of the cervical area gave the lowest and most consistent values of 17-OHCS in plasma. The guinea pigs were handled gently at all

times prior to sacrifice. The clamping procedure was executed rapidly with an instrument exerting sufficient force to immediately stop pulmonary ventilation, general motor activity and blood flow to and from the brain. Plasma 17-OHCS in guinea pigs processed in this fashion ranged from less than 1 to 26 μg./100 ml.; 17-OHCS were not found in the bile of animals with such low plasma levels (8). Even when blood was obtained within a few seconds after a quick cranial blow, the 17-OHCS values were often elevated, occasionally reaching 95 μg./100 ml. When the cervical area was clamped within 15 to 45 seconds after a cranial blow the 17-OHCS of plasma ranged from 29.7 to 83.1 μg./100 ml.

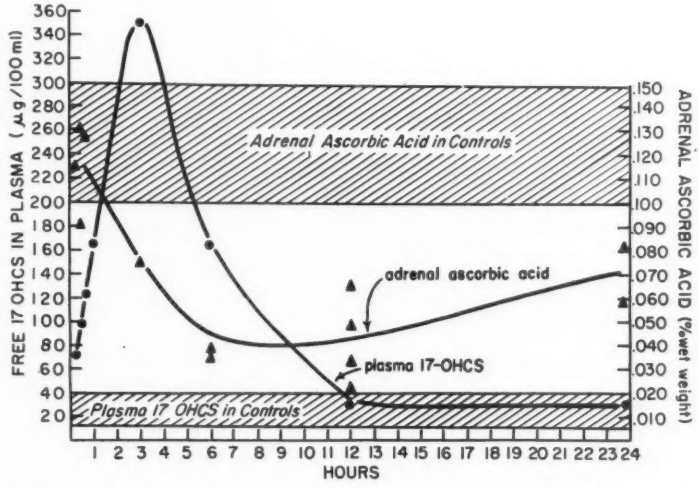
FREE 17 OHCS IN PLASMA (μg/100 ml)
ADRENAL ASCORBIC ACID (%wet weight)
Adrenal Ascorbic Acid in Controls
adrenal ascorbic acid
plasma 17-OHCS
Plasma 17 OHCS in Controls
HOURS

FIG. 1. Plasma levels of 17-OHCS and adrenal ascorbic acid concentrations in guinea pigs after the intravenous administration of bacterial polysaccharide (1 mg./100 gm. body weight).

17-OHCS in Plasma and Bile after Bacterial Polysaccharide

The intravenous injection of a relatively large amount (1,000 μg./100 gm. body weight) of polysaccharide led to a rapid elevation of 17-OHCS in plasma, reaching a maximum in 3 to 6 hours, then decreasing toward the control range in 12 to 14 hours (Fig. 1). Smaller quantities (12–110 μg./animal) of polysaccharide gave less elevation of plasma 17-OHCS and a shorter period of response (Fig. 2). The 17-OHCS increased in the bile and after the first or second hour were higher than those in the plasma (Table 1). When the plasma 17-OHCS declined to the control range, 17-OHCS were no longer present in the bile. After 2, 3, or 5 daily injections of polysaccharide the 17-OHCS in plasma returned to control values in about 12 hours (Fig. 3, Table 1).

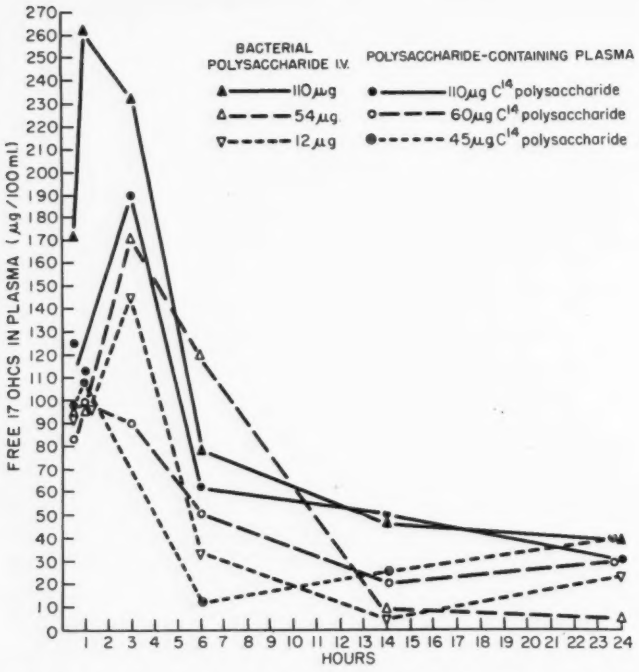

FIG. 2. Plasma levels of 17-OHCS in the guinea pig following the intravenous administration of bacterial polysaccharide and of guinea pig plasma containing C^{14} labeled polysaccharide.

Adrenal Ascorbic Acid

Following a single intravenous injection of polysaccharide the adrenal ascorbic acid concentration declined somewhat inversely to the plasma 17-OHCS values (Fig. 1). While the 17-OHCS in plasma returned to control

TABLE 1. 17-HYDROXYCORTICOSTEROIDS (μg./100 ml.) IN PLASMA AND BILE AFTER THE ADMINISTRATION OF ALKALINE-EXTRACTED K. PNEUMONIAE POLYSACCHARIDE COMPLEX

Amount of polysaccharide injected	Hours after injection											
	½		1		3		6		14		24	
	Plasma	Bile	Plasma	Bile	Plasma	Bile	Plasma	Bile	Plasma	Bile	Plasma	Bile
1 mg./100 gm.* body weight	98.8	88.0	136.0	100.0	216.0	346.0	202.0	361.0	32.0	10.0	19.0	0
.054 mg.* per animal	93.7	83.0	94.3	56.0	170.0	385.0	119.2	232.0	8.4	—	6.0	0
.012 mg.* per animal	91.0	78.0	96.4	61.0	143.7	242.0	34.2		4.2	0	26.0	0
1 mg./100 gm.† body weight			93.2 140.5	132.0 141.0	246.7 245.1	184.0 213.0	344.6 181.3	423.4 536.3			46.8 43.0	0 0

* One injection of bacterial polysaccharide.
† These animals were pretreated with 1 mg./100 gm. of polysaccharide per day for two consecutive days and given the standard dose of 1 mg./100 gm. on day 3.

values in about 12 hours, the adrenal ascorbic acid did not usually return to the control range until the second day. Repeated daily injections of polysaccharide decreased the adrenal ascorbic acid for more prolonged periods; after 5 daily injections of polysaccharide, the adrenal ascorbic acid remained depressed for several days (Fig. 3).

The Effect of Polysaccharide-Containing Plasma

The polysaccharide remaining in the blood 1, 3, or 6 hours after its injection had less ability to produce an elevation in plasma 17-OHCS than a

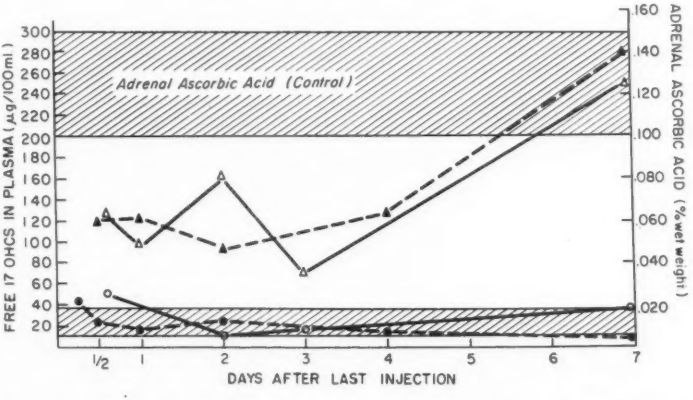

FIG. 3. Plasma levels of 17-OHCS and adrenal ascorbic acid concentrations in guinea pigs after the daily administration of bacterial polysaccharide for 2 or 5 consecutive days.

△ Adrenal ascorbic acid after 2 daily injections of bacterial polysaccharide.
○ Plasma 17-OHCS after 2 daily injections.
▲ Adrenal ascorbic acid after 5 daily injections of bacterial polysaccharide.
● Plasma 17-OHCS after 5 daily injections.

comparable amount of the original polysaccharide (Fig. 2). No plasma 17-OHCS or adrenal ascorbic acid responses occurred when plasma from control guinea pigs was injected (Table 2).

ACTH Response After Bacterial Polysaccharide

The intravenous administration of ACTH at 6, 9 and 12 hours after the injection of the polysaccharide produced a marked increase in plasma 17-OHCS (Fig. 4). This indicates that adrenal 17-hydroxysteroidogenesis can occur even when relatively large amounts of polysaccharide are circulating in the plasma and do not maintain high 17-OHCS levels.

Plasma Clearance of Different Quantities of Polysaccharide

The various quantities of polysaccharide were cleared from the plasma at rates comparable to that of the large dose indicated in Figure 5. Be-

TABLE 2. PLASMA 17-OHCS AND ADRENAL ASCORBIC ACID IN NORMAL ANIMALS GIVEN 0.5 ML. PLASMA FROM GUINEA PIGS GIVEN A SINGLE INJECTION (1 MG./100 GM.) OF *K. pneumoniae* POLYSACCHARIDE COMPLEX 1, 3, AND 6 HOURS PREVIOUSLY

	Hours after plasma injection											
	1		3		6		14		24		72	
	Plasma 17-OHCS μg./100 ml.	Adrenal ascorbic acid %	Plasma 17-OHCS μg./100 ml.	Adrenal ascorbic acid %	Plasma 17-OHCS μg./100 ml.	Adrenal ascorbic acid %	Plasma 17-OHCS μg./100 ml.	Adrenal ascorbic acid %	Plasma 17-OHCS μg./100 ml.	Adrenal ascorbic acid %	Plasma 17O-HCS μg./100 ml.	Adrenal ascorbic acid %
0.5 ml. normal plasma	13	—	24	.105	—	.132	5	.156	2	.127		
0.5 ml. plasma from animals given bacterial polysaccharide, plasma obtained at:												
1 hr.	88	.062	204	.052	249	.057	53	.067	38	.067	16	.190
	113	—	212	.114	170	.073	75	.082	29	—		
			196	—	65	—	50	—				
3 hr.	96	.106	126	.065	115	.049	50	.060	44	.059	12	.171
	103	—	89	—			16	—	29	—		
6 hr.	156	.062	202	.041	163	.040	15	—	39	—		
	152	.100	125	.117			23	—				
	110	—										

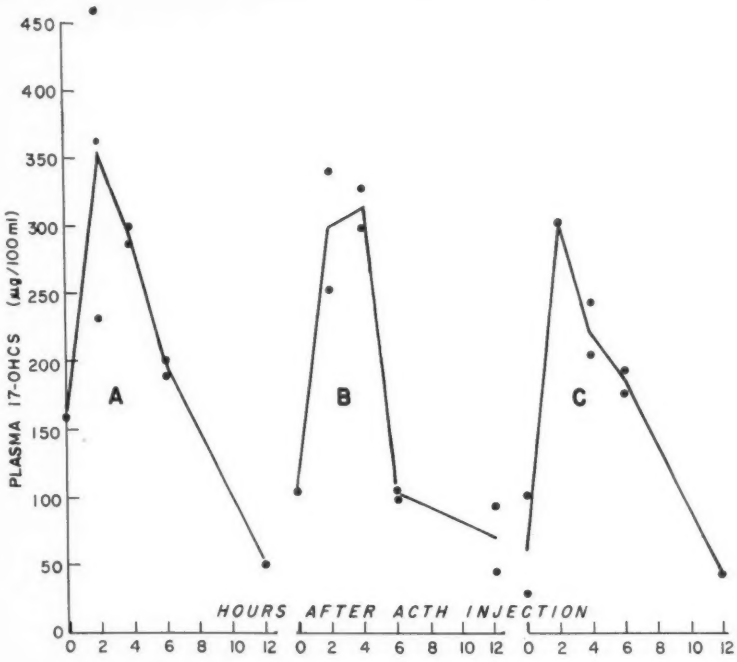

FIG. 4. Plasma levels of 17-OHCS in the guinea pig following the intravenous administration of 0.5 I.U. ACTH/100 gm. body weight.

A. 6 hours after the administration of 1 mg./100 gm. body weight bacterial polysaccharide.

B. 9 hours after the administration of 1 mg./100 gm. body weight bacterial polysaccharide.

C. 12 hours after the administration of 1 mg./100 gm. body weight bacterial polysaccharide.

tween the 2nd and 24th hour, the exponential clearance of the polysaccharide from plasma was 14.7% of the plasma level per hour.

DISCUSSION

Following the administration of gram-negative bacteria or their polysaccharide extracts, increased adrenal activity has been observed in man and the guinea pig by numerous investigators (9–12). The bacteria and their products probably act through an increased discharge of ACTH. The present studies show that in the guinea pig the plasma 17-OHCS elevation is not maintained for more than 12 to 14 hours after the administration of bacterial polysaccharide although the plasma contains ample quantities of the polysaccharide at this time. Rosenfeld's studies of perfused calf adrenal glands (13) suggest that bacterial polysaccharides may act directly

upon the adrenal gland to decrease steroidogenesis, either with or without ACTH in the perfusate. The present studies, however, indicate that the guinea pig adrenal is not "exhausted" or "refractory" after bacterial polysaccharide injection since ACTH induces a plasma 17-OHCS elevation at 6, 9 or 12 hours after the administration of the polysaccharide (Fig. 4). In addition, the third daily injection of polysaccharide evokes a strong 17-OHCS response (Table 1). In order to explain the subsidence of 17-

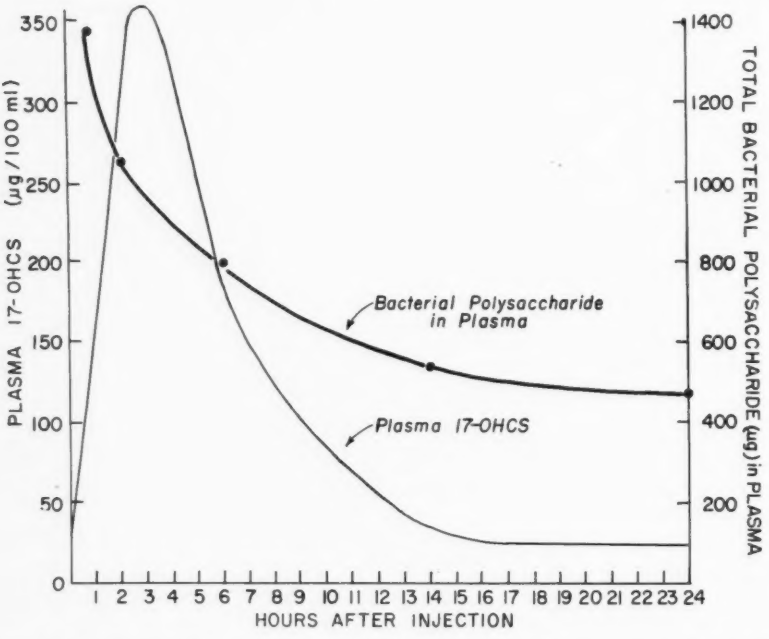

FIG. 5. Clearance of C^{14} bacterial polysaccharide from the blood stream of the guinea pig in relation to the average plasma 17-OHCS concentration curve after intravenous injection of bacterial polysaccharide.

hydroxysteroidogenesis while polysaccharide is still present in the blood plasma (Fig. 5) further studies on the physicochemical changes in the circulating polysaccharide and its influences on the pituitary-adrenal axis are necessary.

Unpublished observations (14) indicate that the polysaccharide remaining in plasma for several hours is not greatly altered since it retains the haptenic properties and tissue affinities of the original polysaccharide. The slower initial rate of disappearance from the blood and the diminished capacity to evoke 17-OHCS elevation in plasma are probably an indication of a physicochemical change in the bacterial polysaccharide. This may

be due to a combination with plasma constituents. LeQuire (15) observed that the thermogenic effect of *E. typhosa* vaccine and of purified pyrogens of *A. cloacae*, *Ps. aeruginosa* and *E. typhosa* was augmented by suspension in citrated but not heparinized plasma. Grant and Whalen (16) postulated the formation of an "endogenous" pyrogen through the interaction of a *Proteus vulgaris* pyrogen with plasma, resulting in a chemical or physical modification of the injected material.

The decrease in adrenal ascorbic acid after one or several injections of bacterial polysaccharide is more prolonged than the elevation of the plasma 17-OHCS in the guinea pig. In the rat, Wexler and co-workers (12) have observed that the administration of Piromen, a *Pseudomonas* polysaccharide, will give a more prolonged depression of adrenal ascorbic acid than seen following the administration of ACTH. The bacterial polysaccharide may, therefore, directly interfere with mechanisms governing adrenal ascorbic acid concentration in different animal species. The more prolonged depression of adrenal ascorbic acid in the guinea pig than in the rat may be due to the inability of the former to synthesize ascorbic acid. The relationship between steroid biosynthesis and adrenal ascorbic acid still awaits explanation. If a positive correlation exists, shifts in adrenal ascorbic acid concentration may reflect other parameters of "stress" metabolism than those pertinent to the production of 17-OHCS.

Does tissue injury result from the relatively high levels of bacterial polysaccharide in the plasma after the termination of the 17-OHCS response? Engorgement of the adrenal, formation of bile precipitate in the gall bladder, accumulation of ascitic fluid and even death have been reported between 6 and 24 hours after the injection of the more toxic bacterial polysaccharides (5). In the guinea pig the adrenal cortex accumulates a marked amount of C^{14} from labeled polysaccharide and attains a maximum concentration of C^{14} less rapidly than other organs or tissue (1, 2). A high plasma concentration of 17-OHCS will not abolish the C^{14} uptake by the gland since such an uptake occurs not only during the initial 6 or 12 hours when the 17-OHCS are high but also with the sustained high levels of 17-OHCS resulting from the treatment with either cortisone or cortisone plus ACTH (17). Our observations are, therefore, compatible with the entrance of labeled moieties from the polysaccharide into the adrenal cortex regardless of the concentration of circulating 17-OHCS.

Could the joint lesions induced by *K. pneumoniae* polysaccharide be due to the presence of circulating polysaccharide after the subsidence of the plasma 17-OHCS elevation? Histologic studies indicate that the metachromatic, PAS-negative material, apparently hyaluronic acid or its complex with protein, initially decreases and later increases in the joints of guinea pigs given a single injection of the polysaccharide and daily subcutaneous injections of ACTH and/or cortisone (17). Since daily injections of either ACTH or cortisone alter the metachromatic material in the joint

space it would appear that either repeated or sustained elevations of plasma 17-OHCS may be of significance. Elevated plasma 17-OHCS could modify the joint lesions by altering a) the distribution of polysaccharide, b) the function of the synovium, or c) the properties of synovial fluid.

Seven or more daily injections of ACTH plus cortisone, but neither alone, lead to moderate quantities of ascitic fluid (3). The administration of alkaline-extracted polysaccharide occasionally produces a mild amount of ascitic fluid while the toxic, acid-extracted polysaccharide regularly produces a more marked, but transient, accumulation of ascitic and retroperitoneal fluid 6 to 12 hours after injection (5). When animals receiving ACTH and cortisone daily are given a single intravenous injection of non-toxic alkaline-extracted polysaccharide, a pronounced accumulation of ascitic fluid develops and persists. Since the toxic acid-extracted and the relatively non-toxic alkaline-extracted polysaccharide both give similar plasma 17-OHCS responses (14) but differ in the production of ascitic fluid, there are significant factors besides the plasma 17-OHCS in this phenomenon.

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THYROIDAL METABOLISM OF DIIODOTYROSINE

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ABSTRACT

The deiodination of radiodiiodotyrosine by animal tissues has been studied and quantitated by paper chromatography. The thyroid and liver were active, the kidney relatively inactive, in deiodination. Slices and mitochondria were more effective than homogenates or supernatants. Inhibition occurred with extremes of temperature and pH, added p-chloromercuribenzoate, mercuric chloride, 2-methyl 1,4-naphthoquinone and iodoacetate. Many enzyme inhibitors (including cyanide, azide, fluoride, methylene blue) compounds known to effect thyroid function (TSH, KSCN, iodide, PTU, tapazole, resorcinol, sulphanilamide), other iodinated compounds (BHDB, Priodax, Teridax, Telepaque, thyroxine, triiodothyronine, 3,5-diiodothyronine) have no effect on deiodination. Carrier diiodotyrosine and monoiodotyrosine at $10^{-3}M$ inhibit deiodination of radiodiiodotyrosine. The products of deiodination are iodide and, presumably, tyrosine. There is no accumulation of monoiodotyrosine except in the presence of iodoacetate. Iodide is released from the slide into the media. The thyroidal enzyme seems to be specific for iodinated tyrosines, not thyronines or other similar compounds. If monoiodotyrosine is formed, its deiodination is not rate limiting.

THE dehalogenation of some halogenated aliphatic (1) and aromatic (2) hydrocarbons is catalyzed by tissue slices, homogenates and extracts. *In vitro*, the liver, thyroid and kidney of the dog deiodinated diiodotyrosine (2). Liver was the most active at 37° C with an optimal pH of 7.7–8.0. Extremes of temperature and pH were inhibitory but anerobic conditions made no difference. Roche, *et al.* (3, 4) extended these observations, with sheep and dog tissues, to include monoiodotyrosine and thyroxine. The thyroid of the sheep was the most active in deiodination of monoiodotyrosine and diiodotyrosine; liver, kidney and heart were less active. Slices and extracts were effective but deiodination was inhibited by boiling. Thyroxine, however, was only slightly deiodinated, if at all. The authors postulated that the thyroid conserved iodide essential for thyroxine synthesis by deiodinating metabolically inactive monoiodotyrosine and diiodotyrosine released by the intrathyroidal proteolysis of thyroglobulin. Michel (5) states that "almost all of the iodine entering the thyroid gland leaves it in the form of hormones after one or more passage in thyroglobulin." The possibility exists, however, that significant amounts of inorganic iodine may leave the gland.

Further studies on the thyroidal deiodination of diiodotyrosine and the

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effects of various physical and chemical agents on deiodination are presented here.

MATERIALS AND METHODS

Fresh tissues were obtained at the abattoir and iced. Slices (about 0.5 mm. thick) or an homogenate of 250–300 mg. of tissue was placed in 1 cc. of Krebs-Ringer phosphate medium buffered at pH 7.8 to which approximately 3 μc. of carrier free diiodotyrosine labelled with I^{131} had been added. Control tubes were without tissue or with boiled tissue. Various compounds were added in concentration usually of $10^{-2}M$ or $10^{-3}M$. The tubes were placed in a constant temperature (37° C) bath for twenty-four hours (shaking did not influence the results). An aliquot (8λ) of medium was then placed on strips, chromatographed in an ascending system of butanol-acetic acid-water, (75–10–15) and both radioautographed and counted on a strip counter. (Actigraph, Nuclear-Chicago) Planimeter measurements were made for quantitation of the Esterline-Angus recording. In most of the experiments the medium contained potassium thiocyanate and propylthiouracil to prevent the uptake by the thyroid of iodine. The effect of various compounds administered *in vivo* was studied by feeding rats diets (Ken-L-Biskit) containing the compounds for two to three weeks. The thyroids were then removed and treated as described above.

RESULTS

The percentage of radiodiiodotyrosine deiodinated by slices of sheep or cow thyroid varied between 20 and 100. In some instances a spot with the R_f of monoiodotyrosine was present, but the large majority of radioactivity was iodide and diiodotyrosine. In addition, material was occasionally noted (particularly in the homogenate experiments) with an R_f of thyroxine, or triiodothyronine, or of unidentified material which stayed at the origin or ran close to the front. None of these compounds was definitely identified, but the formation by tissue of thyroxine or triiodothyronine in the presence of propylthiouracil seemed unlikely. Products of complex formation or iodination of peptides or proteins, or various products of oxidation or reduction and of deamination or decarboxylation may have been formed.

Deiodination was significant (about 50% of final value) at three and six hours but further deiodination occurred between six and twenty-four hours (Fig. 1). Little sensitivity to changes in pH was noted but with pH's of 3 or 11 pronounced inhibition was observed. Boiling the slices for one minute prior to the addition of diiodotyrosine completely inhibited deiodination. Incubation at 5° C, 25° C and 60° C inhibited deiodination, (Fig. 2), whereas anaerobic incubation (nitrogen atmosphere) had slight, if any, inhibitory effect. Doubling the amount of tissue from 250 mg. to 500 mg. per cc. increased the amount of deiodination, whereas increasing the quantity of medium twofold had no effect on the deiodination of diiodotyrosine. The slices retained their ability to deiodinate diiodotyrosine after vigorous shaking for an hour in K-R-P buffer. The buffer, in which the slices had been shaken, gained only a slight ability to deiodinate diiodotyrosine. Some deiodination occurred in Krebs-Ringer-phosphate

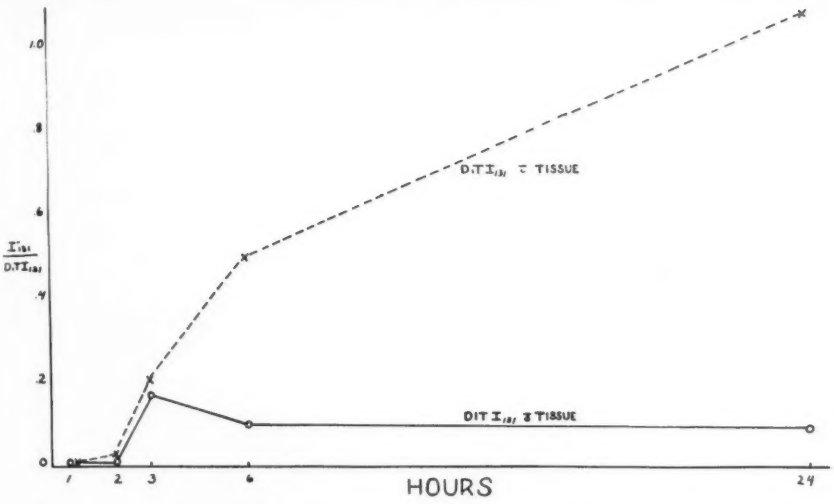

FIG. 1. The ratio of iodide-131 to diiodotyrosine-131 after incubation of diiodotyrosine (about 3 μc.) in K-R-P medium with (dotted line) and without (solid line) added beef thyroid slices. Fifty percent of the deiodination occurs in 6 hours.

medium without calcium chloride or magnesium sulfate or sodium chloride. The addition of glucose to the medium made no difference but plasma reduced deiodination. Slices, washed repeatedly with saline after a twenty-four hour incubation, lost radioactivity (Fig. 3) so as to suggest that there

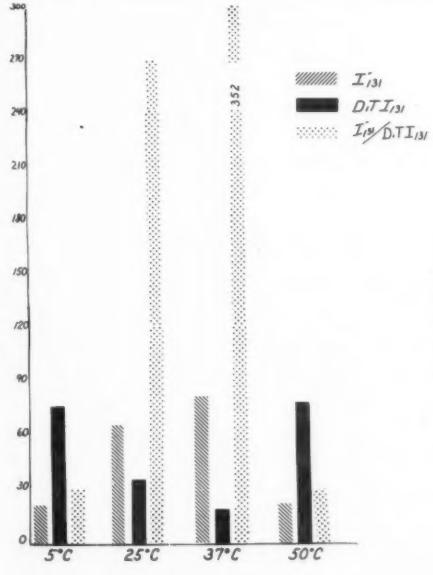

FIG. 2. The effect of temperature on the deiodination of diiodotyrosine by beef thyroid slices, plotted as the ratio $\times 100$ of the amount of the radioactive compound to the total radioactivity of the strip (except I^{-131}/DIT 131).

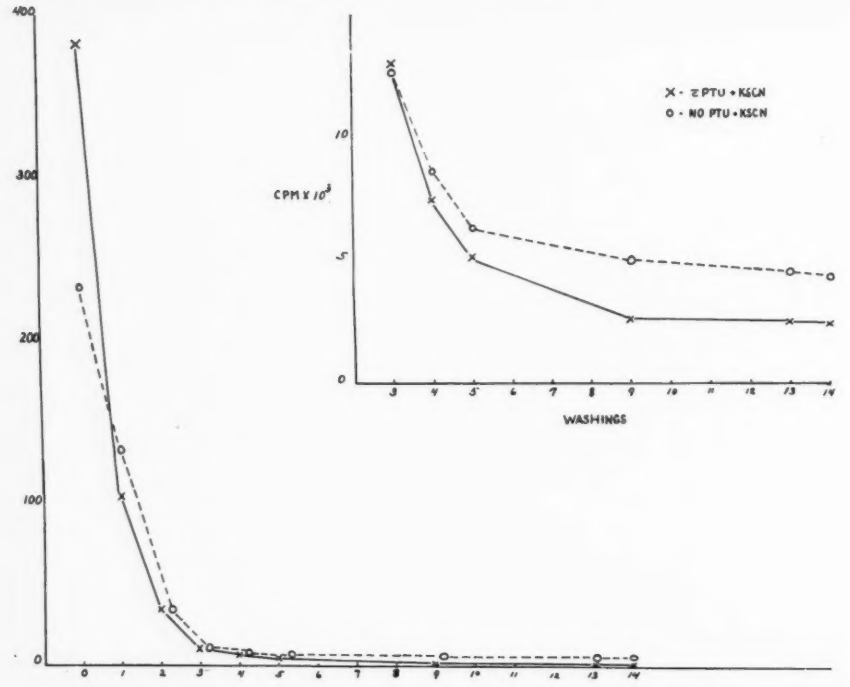

FIG. 3. The effect of repeated washing with isotonic sodium chloride of thyroid slices after 24 hour incubation with radiodiiodotyrosine on residual tissue radioactivity expressed as counts per minute remaining at the end of a particular wash (0–14). The difference between the two lines from wash 5 to 14 presumably represents labelled thyroglobulin. This material was so inactive that after proteolysis, it could not be identified by chromatography. There is no evidence to suggest that propylthiouracil (PTU) or KSCN blocks uptake of diiodotyrosine.

is little, if any, binding (by bonds which cannot be broken with saline) of diiodotyrosine or any of the iodinated products of deiodination by the slice. So little radioactivity remained after washing that it could not be identified by chromatography. The slightly higher residual in slices not blocked by propylthiouracil and thiocyanate suggests that a small amount of thyroglobulin was formed.

The thyroids of the cow and sheep were equally active, but kidney tissue was only slightly active in deiodination. The liver of the cow was the most effective tissue (Fig. 4). Whole homogenate and its supernatant (2000 rpm) deiodinated diiodotyrosine slightly, a mitochondrial preparation (homogenate centrifuged in 0.25% sucrose at 16,000 rpm.) was less active than whole tissue slices (Fig. 5) (both incubated in 0.25% sucrose, which somewhat inhibits the deiodination of diiodotyrosine by normal slices).

The addition to the medium of various compounds known to effect thy-

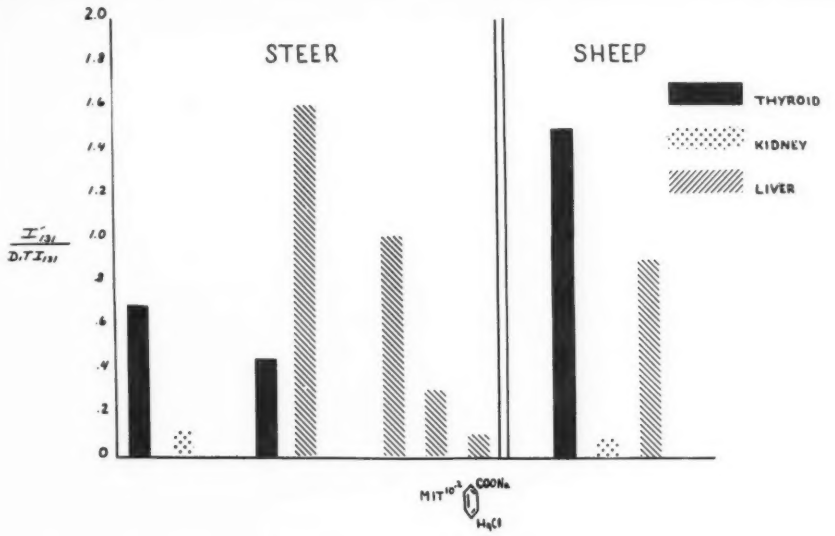

FIG. 4. The extent of deiodination of diiodotyrosine by sheep and steer thyroid, kidney and liver slices expressed as the ratio of radioiodide to radiodiiodotyrosine. Carrier monoiodotyrosine and p-chloromercuribenzoate inhibit the activity of liver slices. Each grouping of columns can be compared only to itself.

roid function did not influence deiodination of diiodotyrosine by thyroid tissue: potassium thiocyanate, propylthiouracil, tapazole, iodide, resorcinol, sulfanilamide, cortisone, estrone. Thyroid stimulating hormone (in amounts varying between 1 mμ/ml. -10^4 mμ/ml.) had no effect on dei-

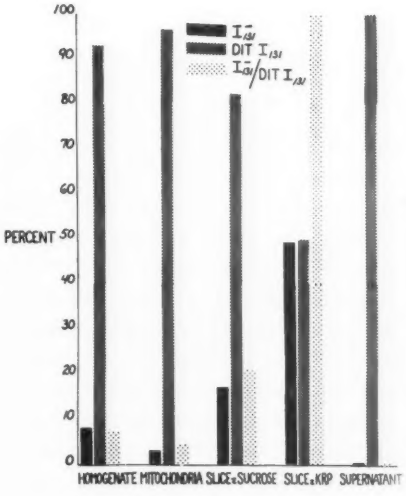

FIG. 5. The comparative ability of homogenates, mitochondria, slices and supernatant preparations of beef thyroid to deiodinate diiodotyrosine expressed as the ratio of the radioactive product to total radioactivity $\times 100$ (except I^{-131}/DIT 131).

odination at three or twenty-four hours. The anti-thyroxine compound n-butyl parahydroxy, 3,5-diiodobenzoate did not inhibit deiodination. Various other iodine containing compounds had no effect: potassium iodide, *l*-thyroxine, 3,5,3′triiodothyronine (*l*), 3,5-diiodothyronine (*l* and *dl*), Priodax (Schering), Teridax (Schering), Telepaque (Winthrop-Stearns). The ceric sulfate-arsenious acid system for determining the presence of iodine (although the results were not always consistent or clear) did not indicate that Priodax, Telepaque, Teridax or 3,5-diiodothyronine were themselves significantly deiodinated. Monoiodotyrosine ($10^{-2}M$), however, markedly diminished the deiodination of diiodotyrosine and the amount of deiodination of radiodiiodotyrosine was greatly reduced by $10^{-3}M$ carrier diiodotyrosine (Fig. 6).

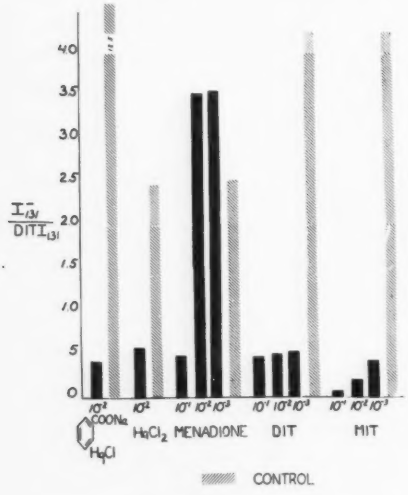

FIG. 6. The inhibition of deiodination of diiodotyrosine by beef thyroid slices by various compounds. The solid black columns represent the results with the indicated inhibiting compound added, the cross hatched columns are simultaneously run controls. There is little if any, inhibition by monoiodotyrosine and diiodotyrosine at $10^{-4}M$.

A number of compounds, including some enzyme inhibitors, had no effect on deiodination: sodium cyanide, potassium ferricyanide, sodium fluoride, sodium azide, hydroquinone, mercaptoacetate, sodium nitroprusside, methylene blue, hydrogen peroxide, versene, arsenious acid, thyronine, potassium thiosulfate.

The results with other compounds, including the heavy metals, were difficult to interpret. Experiments with media, radioiodide or radiodiiodotyrosine and the test compound without tissue indicated the formation of new iodine containing compounds. A significant amount (5–20%) of iodide stays at the origin in the presence of silver nitrate and cupric chloride, a large amount (30–40%) moves near the front with iodoacetate and all the radioactivity moves near the front in the presence of para-chloromercuribenzoate and mercuric chloride. The same reactions could presumably occur with iodide released by the deiodination of radiodiiodotyrosine. In

addition, a small amount of radioactivity (about 5% with Ringers but over 50% with water) from radiodiiodotyrosine stays at the origin in the presence of silver nitrate and cupric chloride, whereas some (5–10%) moves near the front with mercuric chloride and parachloromercuribenzoate (Fig. 7). Some of these findings can be explained by the formation of new compounds (silver iodide, mercuric iodide) or complexes (copper with diiodotyrosine). However, the finding of more iodide in the controls than the sum

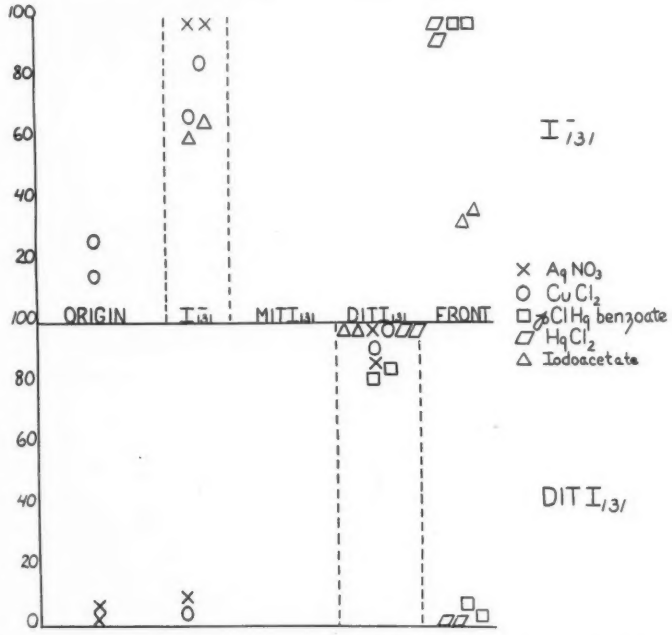

FIG. 7. The effect of various compounds added to medium with radioiodide or radiodiiodotyrosine. No tissue is present. All symbols should be between the two vertical lines; those outside represent material running chromatographically in an unusual manner due to the added compound. The amounts are expressed as percentage of total radioactivity.

of iodide and these other unknown spots in the presence of *p*-chloromercuribenzoate and mercuric chloride indicates an inhibition of deiodination (Fig. 6). The results with silver nitrate and cupric chloride do not clearly indicate a detectable amount of inhibition nor, however, do they rule out such a possibility. Iodoacetate and bromoacetate inhibited deiodination completely at $10^{-1}M$, (Fig. 8a, 8b) partially at $10^{-2}M$ and little, if any, at $10^{-3}M$. An appreciable amount of material with the same Rf as monoiodotyrosine was present when $10^{-3}M$ was used. 2-methyl 1,4-naphthoquinone (Menadione) inhibited deiodination completely at $10^{-1}M$, and occasionally at $10^{-2}M$ (Fig. 6).

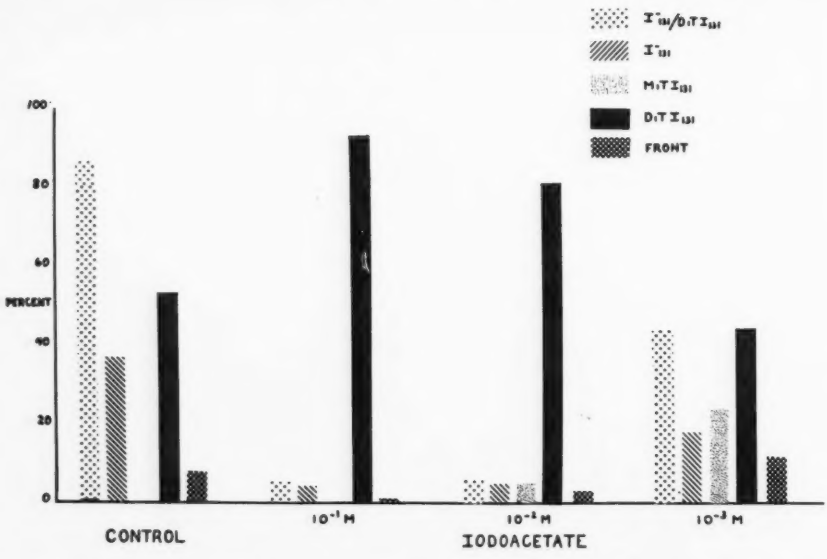

FIG. 8a. The effect of iodoacetate on the deiodination of diiodotyrosine by beef thyroid slices. Note the appearance of significant amounts of monoiodotyrosine as lower concentrations of iodoacetate are used resulting in less inhibition of deiodination. As more iodide becomes available more material runs at the front (some of which is presumably due to the effect of iodoacetate on iodide). The control and $10^{-1}M$ iodoacetate have no measurable monoiodotyrosine. Results are expressed as percent of total radioactivity.

Gross contamination with various microorganisms (including Staph. aureus, A. aerogenes, E. coli, Pseudomonas aeruginosa) in the absence of tissue did not deiodinate diiodotyrosine. *In vivo* studies with rats on diets for 3 weeks of propylthiouracil (0.1%), iodide (1 mg./10 gm.), propylthiouracil plus iodide, estrone, 3,5-diiodo-*dl*-thyronine (500 μg./10 g.), thyroid powder (0.1%) resulted in little if any change in deiodinating ability of the thyroid.

DISCUSSION

The *in vitro* deiodination of diiodotyrosine (2) by animal tissue has been confirmed. The thyroid and liver of the cow and sheep were active, whereas the kidney was inactive. Mitochondria, whole homogenates and supernatants were not so effective as tissue slices. Homogenization and chromatography of the washed slice indicates that diiodotyrosine has either entered or adhered to the tissue and the appearance of iodide in the medium indicates deiodination. This iodide is not bound by the tissue since repeated washings easily remove almost all the radioactivity. Experiments in which propylthiouracil and potassium thiocyanate were omitted indicated an increase in iodide in the medium to a similar extent.

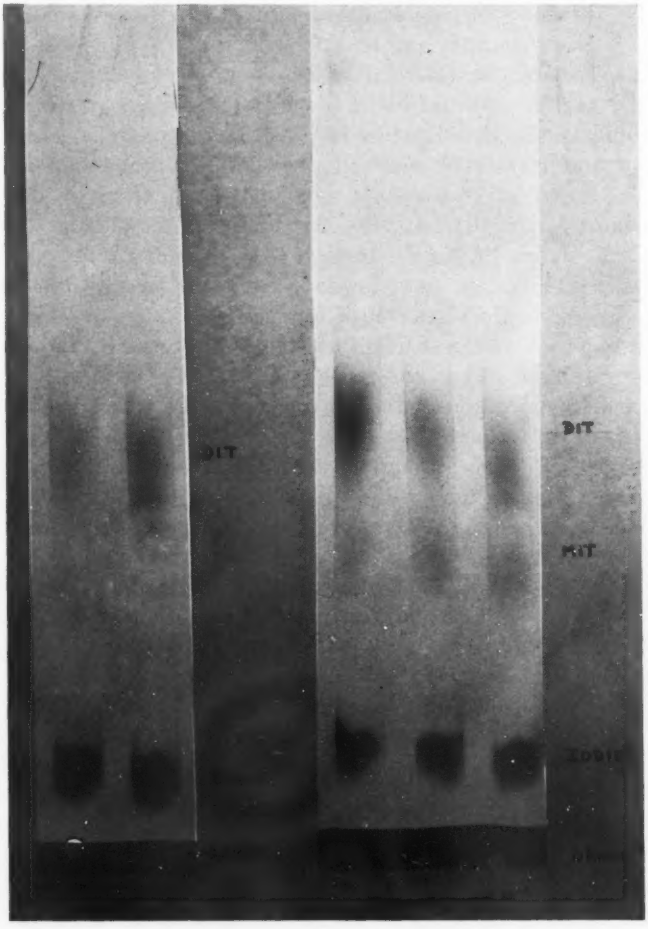

FIG. 8b. Radioautograph of chromatographic strips indicating the appearance of monoiodotyrosine, in the presence of iodoacetate, during the deiodination of diiodotyrosine by beef thyroid slices.

These findings suggest that, in the *in vitro* system used, the iodide released by the deiodination of diiodotyrosine is not necessarily retained by the tissue. The finding by Roche and Michel (4) of labelled thyroxine may be explained by the subsequent uptake from the medium, in the absence of propylthiouracil, of iodide released from diiodotyrosine. If the results of the present study can be applied *in vivo*, the hypothesis advanced by Roche and Michel (4) that the thyroid conserved iodide released by the deiodination of iodinated tyrosines for the synthesis of thyroxine may not hold for all such iodide. Indeed, the thyroid may release a large proportion of the iodide resulting from deiodination of diiodotyrosine.

Tong and Taurog (6) have indicated that the thyroid metabolizes diiodotyrosine by deiodination. The present study confirms this although a small amount of decarboxylation or deamination may be undetected. The final products of deiodination are, presumably, tyrosine and iodide. Unless simultaneous deiodination of the 3 and 5 positions occurs a monoiodo compound must be formed. Very small amounts of monoiodotyrosine, if any, were noted, suggesting that the deiodination of monoiodotyrosine is not rate limiting. In the presence of iodoacetate $10^{-1}M$ and $10^{-2}M$ little deiodination of diiodotyrosine occurred, but at $10^{-3}M$ deiodination occurred and a significant amount of monoiodotyrosine was noted. Iodoacetate may block the deiodination of monoiodotyrosine to a greater extent than diiodotyrosine, suggesting either that a different deiodinating mechanism may be involved or that monoiodotyrosine is more difficult to deiodinate. Diiodotyrosine, however, may be deiodinated in a different form in the presence of iodoacetate.[1]

Many enzyme inhibitors had no effect on the deiodination of diiodotyrosine, including sodium azide, sodium cyanide, sodium fluoride, methylene blue, versene, mercaptoacetate. A nitrogen atmosphere had little effect on deiodination. These findings indicate that neither tissue respiration nor glycolysis need be intact for deiodination. The process is sensitive, however, to temperature and one minute of boiling completely inactivated deiodination. Several inhibitors of sulphydryl groups (*p*-chloromercuribenzoate, mercuric chloride, 2-methyl 1,4-naphthoquinone, iodoacetate and bromoacetate) inhibited the deiodination of diiodotyrosine. In some instances (iodoacetate, bromoacetate) high concentrations ($10^{-1}M$ or $10^{-3}M$) were required and in others (*p*-chloro-mercuribenzoate and mercuric chloride) reactions occurred with iodide and diiodotyrosine. The necessity for unbound sulphydryl groups in deiodinating diiodotyrosine was, therefore, not clearly indicated. Inhibition may have resulted from the oxidative action of heavy metals, or a non-specific effect on the enzyme rather than the specific binding of thiol groups.

It was not clear whether inhibitors blocked the entrance into or adherence to the cell, or the deiodination of diiodotyrosine, or both. Chromatography of homogenized washed slices to which inhibitors had been added

[1] Further studies indicate that the addition of carrier monoiodotyrosine and diiodotyrosine ($10^{-2}M$) to the medium markedly inhibited the deiodination by thyroidal slices of radiomonoiodotyrosine. The addition of carrier diiodotyrosine markedly inhibited, whereas carrier monoiodotyrosine only partially inhibited, the deiodination of radiodiiodotyrosine. The addition of iodoacetate ($10^{-2}M$) inhibited the deiodination of radiomonoiodotyrosine more than the deiodination of radiodiiodotyrosine. There is no difference in the inhibitory effect of mercuric chloride and para chloromercuribenzoate on the deiodination of mono and diiodotyrosine. These findings would suggest that the same enzyme is involved but that monoiodotyrosine is more difficult to deiodinate than diiodotyrosine. However the amount of monoiodotyrosine and diiodotyrosine deiodinated at 1, 3, 6 and 24 hours is similar.

indicated the presence of small amounts of diiodotyrosine similar to the controls. Some diiodotyrosine has, therefore, entered or adhered to the cells, although possibly less than in the control slices. The lack of an accumulation of diiodotyrosine in the inhibited slice does not mean, however, that deiodination may not also have been blocked, since the amount of diiodotyrosine in the slice may be regulated by its intracellular concentration.

Many compounds which affect thyroid function (TSH, iodide, thiocyanate, propylthiouracil, tapazole, resorcinol, sulphanilamide, cortisone, estrone) did not alter the ability of the thyroid slice to deiodinate diiodotyrosine during a twenty-four hour incubation. Michel (5) states that the thyroidal dehalogenase is stimulated *in vivo* by TSH. The present study does not indicate that there is a direct acute effect of TSH on a tissue preparation, although in one preliminary experiment an increase in deiodinating ability of the thyroids of rats injected every two days for three weeks with TSH has been demonstrated.

Other iodinated compounds (thyroxine, 3,5,3′-triiodothyronine, 3,5-diiodothyronine, Priodax, Teridax, Telepaque) were not themselves rapidly deiodinated by thyroid tissue nor did they inhibit the deiodination of diiodotyrosine. Carrier monoiodotyrosine does reduce the deiodination of tracer radiodiiodotyrosine. Thyroidal deiodinating systems can remove iodide from the benzene ring of a tyrosine compound but not of a thyronine derivative or other compounds of similar structure such as: Priodax,[2] Teridax,[2] Telepaque.[4] Diiodotyrosine[5] bears a similarity to these other iodinated compounds which indicates the specificity of the enzyme systems involved. n-butyl p-hydroxy 3,5-diiodobenzoate inhibits the deiodination of thyroxine and triiodothyronine by liver and other tissues (7, 8) but not of diiodotyrosine by thyroid or liver. Diiodotyrosine does not inhibit the deiodination of thyroxine and triiodothyronine by the liver (7). Extrathyroidal tissues, therefore, deiodinate thyroxine and triiodothyronine but not with the same system by which they deiodinate diiodotyrosine. Thyroidal tissue deiodinates monoiodotyrosine and diiodotyrosine, not thyroxine or triiodothyronine. The data does not indicate whether the thyroidal and extrathyroidal systems for deiodinating diiodotyrosine are the same.

Patients have been described recently who presumably have a defect in the thyroidal deiodinating system (9). The present observations do not reveal a physiological inhibitor of thyroidal deiodination, either in short term *in vitro* experiments or more prolonged *in vivo* studies with the compounds tested.

[2] β(4-hydroxy-3,5-diiodophenyl)-α-phenylpropionic acid).
[3] β(5-hydroxy-2,4,6-triiodophenyl)-α-ethylpropionic acid).
[4] β(3-amino-2,4,6-triiodophenyl)-α-ethylpropionic acid).
[5] β(4-hydroxy-3,5-diiodophenyl)-α-aminopropionic acid).

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NOTES AND COMMENTS

DELAYED THYROID RESPONSE TO SERUM FROM THYROTOXIC PATIENTS

ABSTRACT

In mice previously injected with I^{131}, standard thyrotropin produced a maximal increase in circulating radioactivity after a 2-hour interval. A delayed response, similar to that described by Adams and Purves (2, 3) was obtained with certain sera. Thirty-one tests were carried out on sera from 23 subjects. Two pituitary extracts were also tested. A delayed thyroid response was found with sera from 9 of 11 thyrotoxic patients and with sera from 3 patients not thyrotoxic. Sera from 4 myxoedematous, 2 euthyroid and 2 thyrotoxic individuals gave no delayed response; neither did the pituitary extracts. The possibility that this delayed response is of some significance in the aetiology of thyrotoxicosis or is merely a toxic effect of the sera is discussed.

In 1955 Adams and Purves (1) published details of a highly sensitive assay for thyrotropin which was able to detect the serum concentration of thyrotropin in various clinical states. Subsequently they reported an unusual delayed assay response obtained with the sera of certain patients with thyrotoxicosis (2, 3).

A modification of the Adams and Purves assay technique (4) was used to study this unusual effect.

METHODS

Sera and other solutions to be tested were administered intravenously to mice previously injected with I^{131}. Thyrotropic effect was measured by the percentage increase in circulating radioactivity. This increase was found to be maximum at two hours after an injection of U.S.P. Thyrotropin Reference Standard. For the present investigation, radioactivity was measured in blood specimens obtained at 2 hours, 12 hours and 24 hours after an injection of the test substance, as well as prior to injection.

The various sera tested were refrigerated at 4° C until shortly before injection. The volume injected was 0.5 ml. for each of 6 mice except in the case of nos. 14 and 15 where only 0.2 ml. could be tolerated.

RESULTS

Thirty-one tests were carried out on sera from 23 subjects. Also tested were U.S.P. Thyrotropin Reference Standard (0.15 M.U. in 0.2 ml. isotonic saline) and, because it was not expected to contain thyrotropin, a glacial acetic acid extract of human pituitaries, 0.5 ml. containing 12.5 mg. protein. The acid extract was made slightly alkaline before injection. The results are shown in Tables 1 and 2. Typical representative curves are shown in Figure 1. Curve A. obtained with serum from a thyrotoxic patient was a delayed response similar to that described by Adams and Purves. This response was obtained with sera from 9 of 11 thyrotoxic patients and with the sera from 3 other patients, one exophthalmic but not thyrotoxic, one with ophthalmoplegia possibly resulting from an encephalitis and one apparently normal individual. Sera from 4 patients with myxoedema and from 2 euthyroid individuals, and the two pituitary extracts tested, gave no delayed response. Sera from 2 thyrotoxic individuals also gave no delayed response.

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TABLE 1. RESULTS OF ASSAYS FOR A DELAYED THYROID RESPONSE (POSITIVE RESULTS). WITH THESE RESPONSES A MAXIMUM EFFECT WAS FOUND AT 12 HOURS AND NOT, AS WITH STANDARD THYROTROPIN, AT 2 HOURS

Number	Delayed response. Blood radioactivity at 12 hours, % of initial	Diagnosis and comment
1	109	Thyrotoxic with static exophthalmos
2	152	Thyrotoxic
3	164	Thyrotoxic
4	115	Thyrotoxic with exophthalmos
5	157	Thyrotoxic
6	123	Euthyroid—thyrotoxic 2 years ago
7	178	Thyrotoxic
8	129	Exophthalmic, not thyrotoxic
9	Fresh serum 177 Serum 1 week old 106	Thyrotoxic
10	Fresh serum 237 Serum 6 weeks old neg.	Ophthalmoplegic, possibly on the basis of encephalitis contracted while in contact with chicken pox
11a	737	Myxoedematous, having had over-treatment with propyl thiouracil (P.T.U.) for thyrotoxicosis with exophthalmos
11b	219	Same patient as 11a. Euthyroid 3 days after cessation of P.T.U. therapy
11c	212	Same patient as 11a. Thyrotoxic 5 days after cessation of P.T.U. therapy
12	Fresh serum 261 Serum 2 weeks old neg. Serum 4 weeks old neg.	Thyrotoxic with exophthalmos
13	174	Euthyroid

The same specimen of serum from patients numbered 9, 10 and 12 was tested on more than one occasion. The period of storage appeared to be associated with diminution or complete loss of the delayed response. Of the 2 negative sera from thyrotoxic patients, one was tested fresh and the other after 7 weeks' storage.

Sera no. 14 and 15 were the only sera which the mice would not tolerate in injection volumes of 0.5 ml., a very high immediate mortality resulting from injection of this volume. In the few surviving, a positive delayed response was obtained. With 0.2 ml. injections of serum, the mice were obviously ill, apparently shocked, but lived and did not produce a delayed response. These sera were from myself and the technician who assisted me throughout these investigations.

TABLE 2. RESULTS OF ASSAYS FOR A DELAYED THYROID RESPONSE (NEGATIVE RESULTS). THE BLOOD RADIOACTIVITY AT 12 HOURS WAS LESS THAN THE INITIAL MEASUREMENT

Number	Delayed response	Diagnosis and comment
14	–	Thyrotoxic. Serum 7 weeks old
15	–	Thyrotoxic with exophthalmos. Fresh serum
16	–	Myxoedematous, positive early response
17	–	Myxoedematous
18	–	Myxoedematous, positive early response
19	–	Myxoedematous, positive early response
20	–	Euthyroid
21	–	Euthyroid
22	±	Euthyroid. Serum very toxic to mice, with the few surviving giving a positive delayed response. When 0.2 ml. serum injected, less toxic effect with no delayed response
23	±	
24	–	U.S.P. Reference Standard Thyrotropin 0.15 M.U. Positive early response
25	–	Human pituitary extract. Positive early response

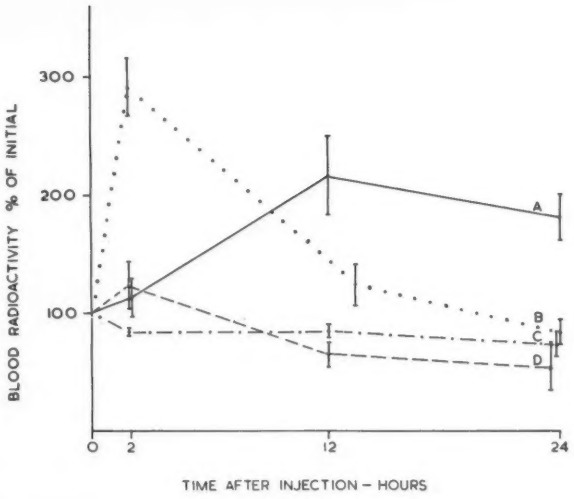

FIG. 1. Representative assay curves. A. Delayed response from the serum of a thyrotoxic patient. B. Early ("normal") response from 0.15 M.U. U.S.P. Reference Standard Thyrotropin. C. No response, from the serum of a euthyroid patient. D. Early response from the serum of a myxoedematous patient. Mean and standard error of the mean of 6 observations shown.

DISCUSSION

The presence of thyrotropic activity in the serum of patients with thyrotoxicosis has been a debatable point for the 25 years during which the results of bioassays have been published. It is likely that the assays of earlier workers were not sufficiently sensitive for their results to be conclusive (5–7). In recent years more sensitive assay techniques have been used to study the problem (8–15). The thyroid status of the patients described in these reports has not always been clearly indicated. Even allowing for this, the published evidence of whether thyrotropin concentration is high, normal or low in the serum of patients with thyrotoxicosis is disconcertingly contradictory.

The delayed response described may be taken as suggestive evidence of thyrotropic activity. Adams (2) found no suggestion that the effect was due to other than a specific thyroid stimulator. Thus, an abnormal thyrotropin may be postulated (3), although other explanations should be kept in mind. For example, a proteolytic effect of the serum giving the delayed response might produce an increase in blood radioactivity due to breakdown of thyroid tissue (16), or there could be stimulation of the pituitary despite the inhibition with exogenous thyroid hormone.

In the assays listed above the reference standard was always a pituitary extract with "normal" thyrotropic activity. The evidence produced here, that some modification of this activity is possible, is in support of the results already reported by Adams and Purves but is still far from conclusive. However, it does raise once more the possibility of an extra-thyroidal aetiology for thyrotoxicosis.

Acknowledgment

I wish to thank Dr. E. B. Astwood for interest in and advice on this study, Miss S. Mothon for considerable technical assistance and Dr. M. S. Raben for the extract of

human pituitaries which was tested. This work was supported in part by grants from the National Institutes of Health, Bethesda, Md. (A-612) and from the American Cyanamid Co. Ltd., Pearl River, N. Y.

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THE EFFECT OF STARVATION AND DEHYDRATION ON RADIOIODINE EXCRETION FOLLOWING INJECTIONS OF I^{131} LABELED THYROXINE[1]

ABSTRACT

Radioiodine excretion in urine and feces was studied in four thyroidectomized-rabbits given daily intravenous injections of I^{131} labeled *l*-thyroxine. Two rabbits were deprived of water for two days, and two rabbits were deprived of food for two days. In both dehydrated and starved rabbits prompt reduction in fecal and urinary radioiodine excretion occurred. This reduction was due, at least in part, to decreased excretion of iodide from degraded thyroid hormone since starved and dehydrated animals were found to have high red cell radioactivity and increase in non-precipitable iodine in the plasma.

During the course of a preliminary study of thyroxine excretion in thyrodectomized rabbits given daily injections of I^{131} labeled *l*-thyroxine unexpected variations in daily excretion of I^{131} were encountered. In some instances decreased I^{131} excretion appeared to follow occasions in which the water-bottle had been allowed to run dry, or had apparently leaked and it seemed reasonable to suppose that the changes noted were at least in part related to dehydration. I^{131} excretion after radiothyroxine injection was studied therefore during a period of deliberately produced dehydration. Since dehydration leads to voluntary food restriction (1) a period of induced starvation was also studied. Although only four rabbits were used in this experiment, the results are so striking that they are believed to be significant and are presented below.

[1] This work was supported in part by United States Public Health Service Grant B834 and by a grant from the Commonwealth Fund.

METHODS

Four adult rabbits of "Dutch" breed weighing between 1.4 and 1.8 kg. were maintained in metabolism cages in a controlled temperature room at 26 ± 1° C, and illuminated by constant photo periods. Purina Guinea Pig Chow and tap water were offered *ad lib* except as noted. Total thyroidectomy was carried out under sodiumpentobarbital anesthesia a week before the start of the experiment. Thereafter a maintenance dose of *l*-thyroxine[2] of 10 μg. was administered daily.

At the beginning of the experiment proper, thyroxine was mixed with chromatographically pure I^{131} labeled thyroxine[3] so that the daily injection of one ml. contained 10 μg of carrier thyroxine labeled with I^{131}-thyroxine, and this was administered intravenously once daily immediately after the end of the 24-hour collection period. In order to standardize the daily injection, the entire lot to be used was divided on the first day and frozen in vials sufficient for one day's therapy. Initially the daily injection contained 2 μC of I^{131}-thyroxine. At the end of each collection period the bladder was emptied manually. Radioactivity counts were made of urine, feces, and blood with appropriate dilutions and constant geometry using a scintillation counter. Correction for radioactivity decay was made by comparison with a standard solution.

RESULTS

Daily excretion of radioiodine in urine and feces in each rabbit are illustrated (Fig. 1–4). Large spontaneous variations in daily excretion were noted in 3 of the 4 rabbits, ranging from 55.4% to 154.4% after the third day of the collection period. As noted above, at least part of the variations appeared to be related to accidental changes in water intake. A two-day period of water deprivation was then imposed on Rabbits *62* and *65*. A two-day period of food deprivation imposed on Rabbits *64* and *66*. Adequate water was allowed starved rabbits, adequate food allowed thirsted rabbits.

The dehydrated rabbits showed striking decrease in total excretion of I^{131}. This was associated with marked decrease in the actual amount of feces and urine. In Rabbits *62* and *65* fecal excretion on the second day of dehydration fell to 0% and 1.9% respectively, as compared with an average of 28.6% and 35.8% in the previous nine days. The first three days were not considered because it takes three days for well hydrated rabbits to come into thyroxine equilibrium. Urinary excretion on the second day of dehydration fell to 15.4% and 16.5% respectively, as compared to an average excretion of 61.1% and 65.7% in the control period.

After water was again given, there was a

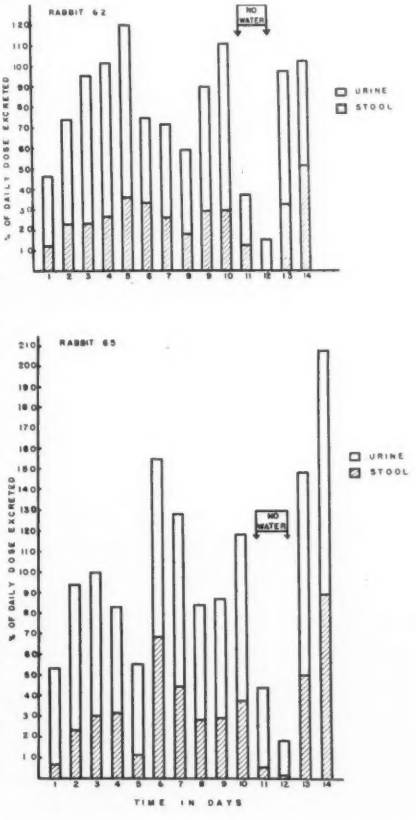

FIG. 1. The effect of a two-day-period of dehydration upon the fecal and urinary excretion of radioiodine by thyroidectomized rabbits receiving daily injections of I^{131}-labeled *l*-thyroxine.

[2] Obtained through the courtesy of Dr. L. D. Bechtol, Travenol Laboratories, Morton Grove, Illinois.

[3] Abbot Laboratories, Oak Ridge, Tennessee.

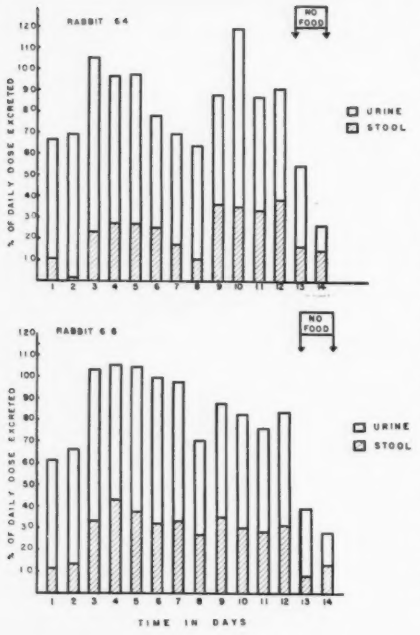

FIG. 2. The effect of a two-day period of starvation upon the fecal and urinary excretion of radioiodine by thyroidectomized rabbits receiving daily injections of I^{131}-labeled *l*-thyroxine.

rise in excretion both in the feces and the urine, which reached 102.2% and 207.5% for single day excretions following rehydration.

The starved rabbits showed similar though less marked decreases in fecal and urinary radioiodine excretion. The excretion of thyroxine after feeding was reinstituted was not followed as no further quantities of labeled thyroxine were available.

In order to gain some idea as to whether the decrease in I^{131} excretion during starvation and dehydration was a consequence of decreased thyroxine degradation, or decreased excretion of thyroxine degradation products, plasma free and protein bound I^{131} was determined after trichloracetic acid precipitation. Red blood cell radioactivity was also determined as a measure of diffusible I^{131} (Table 1). Determinations were made in the dehydrated animals after 53 hours of dehydration and 29 hours of rehydration. In the starved animals determinations were made before and 29 hours after food restriction. In all instances specimens were obtained five hours after the usual daily I^{131}-thyroxine injection. Hemoconcentration was noted in the dehydrated rabbits as compared with the normally hydrated animals. Elevated hematocrit levels fell to the range of the normally hydrated animals within one day of water *ad libitum*.

There are too few observations to evaluate the significance of the level of protein bound I^{131}, but there were no consistent differences between thirsted and non-thirsted animals. More striking, however, was the presence of large amounts of I^{131} in the red cells of dehydrated rabbits which fell markedly after rehydration, and the surprising finding of a similar increase in diffusible I^{131} after only 29 hours of food restriction in the starved animals. Plasma nonprecipitable I^{131} followed the same trend as the red blood cell radioiodine but the magnitude of change was much less.

DISCUSSION

These observations show that complete water or food restriction in rabbits leads to prompt and striking decrease in the excretion in urine and feces of iodide and iodide-containing substances derived from thyroxine. The important question as to whether this represents a decrease in thyroxine degradation rate, or a decreased excretion of the breakdown products of thyroxine degradation cannot be answered by the scanty data presented, but it was noted that the decrease in excretion was associated with large increases of diffusible iodide which suggests that a major effect is a decrease in the excretion of iodide from degraded hormone.

Fecal excretion of I^{131} after the injection of labeled thyroxine in the rabbit normally amounts to about 35% of the total excretion. If enteral excretion in the rabbit resembles that of the rat it may be presumed that the greater part of this is thyroxine or con-

TABLE 1. HEMATOCRIT, PLASMA RADIOACTIVITY, PLASMA NON-PRECIPITABLE IODINE AND RED BLOOD CELL RADIOACTIVITY FOLLOWING RADIO-THYROXINE INJECTION IN DEHYDRATED AND STARVED RABBITS

			Plasma radioactivity		
Rabbit	Condition	Hematocrit %	Counts/ml./min.	% not precipitated by trichloracetic acid	Red blood cell radioactivity (counts/ml./min.)
62	Dehydrated	48	2080	22.1	916
	Rehydrated	40	1290	19.1	0
65	Dehydrated	44	2210	17.6	660
	Rehydrated	36	1335	11.4	222
64	Before Starvation	37.5	1730	11.2	0
	After Starvation	39	1120	17.1	510
66	Before Starvation	38	2110	5.4	0
	After Starvation	36	2040	9.7	845

jugated thyroxine compounds. Water restriction and less strikingly, food restriction, lead to a marked reduction in fecal volume and an associated decrease in fecal radioiodine.

No data are available on the enterohepatic circulation of thyroxine during states of starvation and dehydration but Van Middlesworth (3) has recently demonstrated the importance of fecal mass in the rate of loss of thyroxine from the body of the rat. This observation is possibly pertinent to the mechanism of thyroid inactivity during starvation (cf. 1) since starvation may lead to conservation of available thyroxine supplies through changes in fecal volume. That this is not the only mechanism is suggested by the finding of low serum protein bound iodine in starved rats (1).

Marked reduction in urinary radioiodide excretion was noted following both dehydration and starvation. In association with the piling-up of diffusible iodide in the body this reduction suggests a decrease in renal clearance of radioiodide. Although the renal mechanism for iodide excretion has not been studied in the rabbit, it is apparent from experiments in man (4) and in dogs (5) that iodide excretion is a function of glomerular filtration rate which is presumably decreased in dehydrated rabbits with hemoconcentration. Less apparent is the reason for accumulation of radioiodine in starved animals, since data are not available on the effects of acute starvation on glomerular filtration rate in the rabbit. It should be noted, however, that changes in glomerular filtration are readily produced in the rabbit by emotionally disturbing stimuli (6). It may be that the kidney of the rabbit responds to the stress of starvation in a similar manner.

SEYMOUR REICHLIN

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THE ENDOCRINE SOCIETY

CITATIONS FOR THE 1958 AWARDS

The Endocrine Medal

While the scientific contributions of I. Lyon Chaikoff have by no means been restricted to the field of endocrinology, it is therein that this prolific and many faceted investigator has had the greatest influence. He was born in London, England, July 2, 1902. He grew up, however, in Toronto and graduated from the University of Toronto in 1920 at the age of 18. Under the direction of Professor J. J. R. MacLeod he received an M.A. in 1921 and a Ph.D. in 1924. In this period when the work of Banting and his coworkers burst with such impact upon the field of diabetes he carried out pioneering studies, such as that concerned with keto-acid metabolism which antiquated the concept that "fats burn in the fire of carbohydrate." While in the midst of these studies and teaching in the Physiology Department, he paused long enough to acquire an M.D. degree. In 1930 he joined the faculty of the University of California at Berkeley and was appointed Assistant Professor the following year. In 1939 he was named Associate Professor and has been Professor of Physiology since 1942. In 1941 he was awarded a fellowship of the John Simon Guggenheim Foundation.

From his early interest in the metabolic derangements in diabetes and the natural light that they shed upon normal metabolism, his interests have become so broad as almost to defy characterization. From atherosclerosis to zinc metabolism he has pioneered and paved numerous pathways, using the methods of numerous disciplines and the spectrum of biological material from algae to elephants. It is, indeed, difficult to find an important area wherein he has not ranged during the last two decades: isotope labeling and tracer theory, lipotropic agents, carbohydrate, lipid, protein and mineral metabolism, alcohol, atherosclerosis, radiation-biology, carcinogenesis, nutrition, and metabolic antagonism, to cite only some.

Stemming from his early association with the Toronto investigators has been a continued and productive interest in diabetes mellitus. In 1952 his Harvey Lecture dealt with the metabolic defects in this condition. Although his contributions to our understanding of intermediary lipid, carbohydrate, and protein metabolism in the normal and diabetic would more than suffice to place him in the first echelon of endocrine investigators, his activities have not been limited to that large area.

His observations of thyroid structure, chemistry, and function have over a span of slightly less than twenty years become major stepping stones in the path toward clarification and delineation in this field. Starting with a systematic investigation of iodine metabolism in the thyroid, using the powerful new tool of radioactive iodine and more sophisticated methods

of iodine microchemistry, he has continued to maintain and extend these contributions up to the present time.

In addition to the interest in the thyroid and pancreatic islets his endocrine endeavors have encompassed cytology, oncology, and physiology of the anterior pituitary; the endocrine hypothalamus; lactation; and gonadal and adrenal steroid metabolism and their relation to atherogenesis, fatty liver, and cirrhosis. His work has been characterized not only by high quality, fundamental character, and universal acceptance, but also by its wide diversity of area, interest, technique, and approach.

The Endocrine Society finds it an honor to recognize such a distinguished investigator.

The Ciba Award

Dr. Monte Arnold Greer has been awarded the Ciba Award for 1958 in recognition of 10 years of distinguished research. He was born in Portland, Oregon, on October 26, 1922, and after attending Stanford University and its medical school, began a career of investigation at the age of 24. His aptitude and imaginative approach were quickly recognized and led to a sequence of increasingly important appointments in several institutions, including the New England Center Hospital and Tufts University School of Medicine, the Evans Memorial Hospital and Boston University School of Medicine, the National Cancer Institute and George Washington University School of Medicine, the Radioisotope Service of the Veterans Administration Hospital in Long Beach, California, and, finally, in his own home town to the appointment of Associate Professor of Medicine in the University of Oregon Medical School.

The major accomplishments of Dr. Greer have included the determination of the chemical nature of an antithyroid agent in foods, the control of simple goiter with thyroid administration, and the hypothalamic regulation of anterior pituitary secretion. He has described his carefully conducted experiments on these and related topics in some 50 well-written papers.

The antithyroid action of a large number of foods was surveyed by determining their interference with the accumulation of radioactive iodine by the human thyroid. The potency of the brassica family of plants was noted, and rutabaga was chosen for chemical study. He then participated in the isolation of the antithyroid agent and in its identification as L-5-vinylthiooxazolidone. He has recently isolated and identified the naturally occurring precursor, progoitrin, a glucoside which is enzymatically cyclized to the active oxazolidone when the raw food is crushed. This solution of a long-standing problem contributes importantly to an understanding of the influence of foods on the thyroid gland and of the entire problem of goiter.

Dr. Greer's much-quoted study demonstrating the suppressive effects on thyroid function in normal individuals of orally administered thyroid

extract provided a scientific rationale for the successful use of thyroid to produce regression of non-toxic goiters and thyroid nodules. Additionally, his findings that the thyroid gland of thyrotoxic individuals resisted functional suppression by thyroid administration provided a useful and widely used diagnostic procedure for thyrotoxicosis.

A clear demonstration of the hypothalamic control of thyrotropin secretion resulted from studies on the effects of hypothalamic lesions and the location of the controlling area was described. The goitrogenic effects of antithyroid compounds were eliminated by effective lesions, and the center for thyrotropin control was shown to be different from those concerned with corticotropin and gonadotropin secretion and those related to obesity or emaciation. Dr. Greer was also the first to provide evidence for a "drinking center" in the hypothalamus, and he further showed that the state of persistent estrus due to hypothalamic lesions was corrected by chronic administration of progesterone.

Dr. Greer's impressive achievements in endocrine research, and his continuing productivity make him an outstanding recipient of the Ciba Award.

Upjohn Scholar

Dr. William Peter Upritchard Jackson has been named Upjohn Scholar of the Endocrine Society for 1958 to permit him travel in the United States. Dr. Jackson, born in England in 1919, graduated with honors from Cambridge University and received his medical training there and at the London Hospital. Nine years ago he went to South Africa where he now holds the posts of Senior Lecturer in the Department of Medicine and Fellow of the University of Cape Town, Physician and Deputy Head of the Groote Schuur Hospital, Chief of the Endocrine Clinic and Endocrine Research Laboratories, and Assistant Director of the Council of Scientific and Industrial Research. His contributions to clinical medicine and especially to endocrine and metabolic diseases, prediabetes, calcium and phosphorus metabolism, and nutrition, as well as his broad interests in the out-of-doors, insure that his visits here will be profitable both to him and to us.

Schering Scholar

Dr. Roy Orval Greep, Editor of Endocrinology for the past six years, was born in Longford, Kansas, in 1905, giving prominence to a region which was later to be identified also with Abilene. After graduation from Kansas State College he obtained M.A. and Ph.D. degrees from Wisconsin. From 1935 to 1938 he was Assistant and Instructor in Zoology in the Biological Laboratories at Harvard, and after six years of research at the Squibb Institute of Medical Research returned to head research at Harvard's new School of Dental Medicine. Currently he surmounts the several posts of Professor of Anatomy and Dean of the School of Dental Medicine and

Acting Head of the Department of Anatomy at the Medical School. The broad scope of Dr. Greep's contributions to endocrinology range over the anterior and posterior pituitary hormones, the hormones bearing upon reproduction, and those of the parathyroids, adrenals, and pancreas. He was the first to establish that thyrotropin and luteinizing hormone are separable, that the thyroid can be activated by means other than thyrotropin, that full function can follow transplantation of the anterior pituitary provided the graft is made in the normal position of this gland, and that the adrenal cortex is indeed two anatomically and functionally distinct glands.

Dr. Greep plans to undertake what he describes as "an endocrinologic refueling operation" by visiting Universities, laboratories, and European cities (including Paris) and the Endocrine Society feels it a privilege to contribute to this praiseworthy undertaking by making him the Schering Scholar for 1958.

ERRATUM

In the article by A. J. Lostroh and P. Woodward appearing in ENDOCRINOLOGY *62*(4):498–505, 1958 (April issue), the letter labeling of three figures on page 501 should be changed as follows: C should be labeled E, D should be labeled C, and E should be labeled D.

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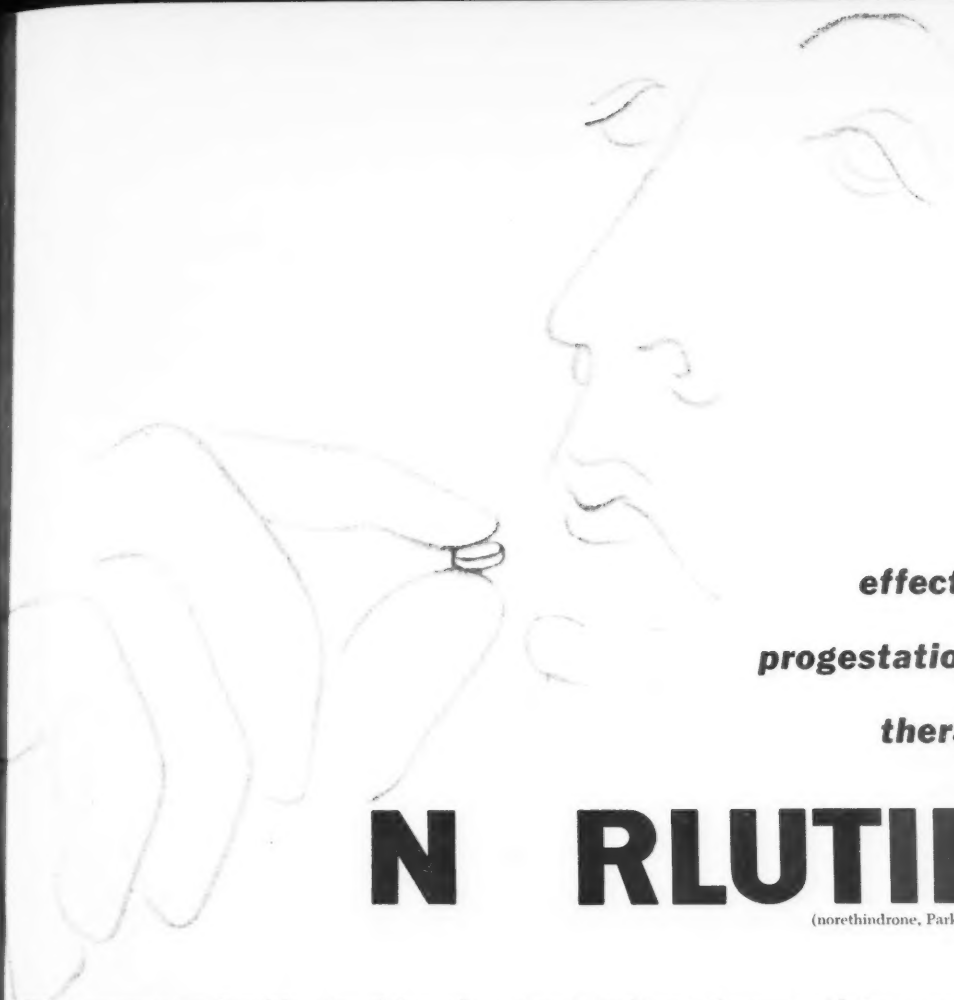

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